The Dissertation Committee for Younghoon Kee certifies that this is the approved version of the following dissertation:

A regulatory mechanism for Rsp5, a multifunctional ubiquitin ligase in *Saccharomyces cerevisiae*: characterization of its interaction with a deubiquitinating enzyme

Committee:

Jon Huibregtse, Supervisor

Dean Appling

Arlen Johnson

Tanya Paull

Scott Stevens

A regulatory mechanism for Rsp5, a multifunctional ubiquitin ligase in *Saccharomyces cerevisiae*: characterization of its interaction with a deubiquitinating enzyme

by

Younghoon Kee, B.S.; M.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fullfilment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2006

Dedication

This dissertation is dedicated to my loving wife, Kyewon, and my parents for their love, encouragement, and unconditional support that they have given me throughout my graduate studies.

Acknowledgement

First, I would like to extend my utmost gratitude to Dr. Jon Huibregtse for allowing me the opportunity to work in his lab and learn how to build a career in science. He has been not only my model as a successful scientific researcher but also a great teacher. I also wish to extend my sincerest thanks to my committee members, Drs. Dean Appling, Arlen Johnson, Tanya Paull, and Scott Stevens for their continuous support and advice during my graduate studies. I would also like to thank all the past and current members of the Huibregtse lab: Sylvie Beaudenon, Cathy Salvat, Melissa Kelley, Nancy Lyon, Anahita Dastur, Hyungchul Kim, Larissa Durfee, and William Munoz for their support and helpful suggestions over the years. I would also like to thank people outside the lab for helpful discussion and suggestions, including Velu Soundarapandian and Jayaram lab members, Changwon, and Zhiwha. I especially thank Velu for his constructive advice and much appreciated discussions during my early stage of the study. I also thank Dr. Klaus Linse for his support on the mass spectrometry analysis. I am also grateful to my friends Jeesun and Juwon, Jeongtae, Jeongchul, Changkeun, Insuk, Sky, Anirvan, Ram, and Lakshmi. Finally, I would like to thank my loving wife, Kyewon, and my lovely daughter, Yewon, for their love and support.

A regulatory mechanism for Rsp5, a multifunctional ubiquitin ligase in *Saccharomyces cerevisiae*: characterization of its interaction with a deubiquitinating enzyme

Publication No._____

Younghoon Kee, Ph.D

The University of Texas at Austin, 2006

Supervisor: Jon Huibregtse

HECT E3 ubiquitin ligases are widely distributed from yeast to human cells and play important roles in many physiological processes. Rsp5, an essential HECT E3 ligase in *Saccharomyces cerevisiae*, is involved in many biological processes, including transcriptional activation, endocytic trafficking, mitochondrial inheritance, and RNA export pathways. Although Rsp5 has been shown to regulate multiple pathways targeting multiple substrates, mechanisms for regulating the biochemical activity of Rsp5 are largely uncharacterized (121, 199). To gain further insight into the regulation of this enzyme, I identified proteins that copurified with epitope-tagged Rsp5. Ubp2, a deubiquitinating enzyme, was a prominent copurifying protein. Rup1, a previously uncharacterized UBA domain protein, was required for binding of Rsp5 to Ubp2 both *in*

vitro and *in vivo*. Biochemical and genetic evidence are consistent with a model that Ubp2and Rup1 antagonizes Rsp5-catalyzed substrate ubiquitination. *In vivo* and *in vitro* experiments showed that Rsp5 and Ubp2 display strong preferences for assembly and disassembly of K63-linked polyubiquitination, respectively. A large fraction of the K63 conjugates in *ubp2*Δ cells bound to Rsp5, and a proteomics approach was therefore used to identify Rsp5 substrates subject to Ubp2 regulation. Two proteins implicated in cell wall integrity, Csr2 and Ecm21, were identified and both proteins were efficiently K63-polyubiquitinated by Rsp5 and deubiquitinated by Ubp2. I have also shown that cell wall integrity is impaired in *rsp5-1* cells and this can be rescued by either *ubp2*Δ or *rup1*Δ mutation, suggesting that the Ubp2/Rup1 complex negatively regulates Rsp5-mediated cell wall homeostasis. Together, these data represent a novel regulatory mechanism for Rsp5 and suggest that similar mechanisms might be utilized by its mammalian homologues. Furthermore, this work provides a basis for studying the mechanism for differential polyubiquitin chain type synthesis by HECT E3 ligases.

Table of Contents

List of Tables

List of Figures

List of Illustrations

List of Abbreviations

a.a.	Amino acids
Ab	antibody
ADCB	L-azetidine-2-carboxylic acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CBP	Calmodulin binding pepetide
CFW	Calcofluor white
CTAP	Carboxyl-terminal TAP
Cys	cysteine
Dex	Dextrose
DUB	Deubiquitinating enzyme
DTT	Dithiothreitol
E1	E1 ubiquitin activating enzyme
E2	E2 ubiquitin conjugating enzyme
E3	E3 ubiquitin ligase
EDTA	Disodium ethylenediamine tetraacetate dihydrate
GST	Glutathione S-transferase
Gal	Galactose
HA	Hemagglutinin
HECT	Homologous to E6AP C-terminus
IgG	Immunoglobulin G
kDa	kilodalton
K0	No Lysine
K48	Lysine 48
K63	Lysine 63
LC/MS	Liquid chromatography/mass spectrometry

ug	microgram
ul	microliter
uM	micromolar
M.W	Molecular weight
NEM	N-ethylmaleimide
NTAP	Amino-terminal TAP
O.D.	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RING	Really interesting new gene
Rsp5	Reverse of spt3 phenotype
SD	Synthetic dropout
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrphoresis
Ub	Ubiquitin
UBA	Ubiquitin associated domain
Ubal	Ubiquitin aldehyde
UBD	Ubiquitin binding domain
UBP	Ubiquitin specific protease
Ubp2	Ubiquitin specific protease 2
UbVS	Ubiquitin vinylsulfone
TAP	Tandem affinity purification
TEV	Tobacco etch virus
Tris	Tris (hydroxymethyl) amino-methane
t.s.	Temperature sensitive
YPD	Yeast extract/peptone/dextrose
YPG	Yeast extract/peptone/galactose

CHAPTER ONE

Introduction

1. 1 Prelude

In the early 1980s, Hershko, Rose, and Ciechanover, the three Nobel Chemistry laureates of 2005, proposed the multi-step ubiquitination hypothesis by isolating the E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases (31, 87). It was proposed that these enzymes work in concert and in a sequential manner to target substrates for ubiquitination. Since this pioneering work, there has been significant advancement in our understanding of the system. This system turns out to be far more complicated than originally appreciated and far more widespread than originally envisioned, as it is now estimated that approximately a quarter of the total proteins in yeast are ubiquitinated (171).

Although ubiquitin was initially isolated during a search for hormones in the thymus that induce differentiation of lymphocytes (73), it took several years to uncover the role of ubiquitin in proteolysis. ATP-dependent proteolysis was first described in work which showed that abnormal proteins are degraded at faster rates than normal proteins in rabbit reticulocyte lysate (58). A year later, it was shown that the components responsible for the proteolysis are biochemically separable into two fractions (APF I and II; ATP-dependent proteolysis factors I and II), with the proteolytic activity of APF1 dependent on APF2 (33). It was later shown that APF1 was ubiquitin (235) and APF2 was the proteasome (234). A surprising and significant observation followed, when it was shown that APF-1 was covalently conjugated to proteins, generating high molecular weight species (32). Since this discovery, a great deal of efforts has been devoted to characterize the mechanisms and functions of ubiquitination.

Although it has been generally accepted that ubiquitination is a mark for destruction of the targeted proteins by proteasome, it has become evident that ubiquitination of proteins also plays non-proteolytic functions. One of the most intriguing current questions in the ubiquitin field concerns the machinery and mechanisms that direct the different regulatory outcomes, which remain largely uncharacterized.

I began my Ph.D. project by characterizing factors that regulate the catalytic activity of Rsp5, an essential HECT domain E3 ligase in *Saccharomyces cerevisiae*. During the course of my research, I have contributed toward understanding how this HECT E3 is regulated by associated factors in cells. Furthermore, my work provides a basis for better understanding the mechanisms and functions of the synthesis of the non-proteolytic K63-linked polyubiquitination.

1.2 Ubiquitinating enzymes

Ubiquitination of target proteins occurs as the result of a cascade of three classes of enzymes (174). The first step of ubiquitination involves a two-step reaction, catalyzed by E1 ubiquitin activating enzyme. First, ubiquitin is activated at its C-terminus by adenylation using ATP, releasing AMP and pyrophosphate. Second, the carboxyl group of activated ubiquitin is subsequently attacked by the active-site cysteine residue of E1, forming an E1-ubiquitin thioester intermediate. The activated ubiquitin is subsequently transferred to the active-site cysteine of E2 ubiquitin conjugating enzymes, in a trans-thiolation reaction. The ubiquitins tethered to the E2s can be directly transferred to the lysine residues of target substrates, or the substrate

ubiquitination can be preceded by a third thioseter bond formation between the ubiquitin and the active-site cysteine residue in the E3. The ubiquitination cascade involving E1, E2, and E3 is known to occur in a sequential manner, as a recent report suggests a model that E2 must dissociate from E1 before it can interact with an E3, by showing that binding of an E2 to E1 is mutually exclusive to binding of E2 to E3 (55). Most organisms contain only one or a few E1 enzymes, a dozen or more E2 enzymes, and a large number of E3 ligases, forming a cascade of the biochemical pathway. E3s are mainly devoted to recognizing individual target substrates, and, therefore, are the most abundant components in the ubiquitin system to cover a wide range of target substrates.

The two major classes of E3 enzymes are the RING (really interesting new gene) E3s (146) and the HECT (homologous to E6AP C-terminus) E3s (105). In general, the RING E3s refer to both the single polypeptide RING domain proteins and the multi-protein complexes that contain the RING-finger domain subunits (174). For simplicity, both classes of RING E3s will be discussed together. There are additional families of proteins that represent minor classes of E3 ligases, such as U-box proteins and PHD domain proteins, as discussed below.

HECT E3

The HECT domain was first discovered in a series of studies on the degradation of p53 tumor suppressor (106, 191, 194). The initial observation showed that the levels of p53 proteins in the high-risk HPV (human pappilomavirus) positive cell lines are very low or undetectable (192). In an *in vitro* study, it was shown that the high-risk E6

proteins of HPV bind to p53 and stimulate the degradation of p53 in the ubiquitin/proteasome-dependent manner (194). Subsequent biochemical studies showed that a cellular protein named E6AP (E6-associated protein) was required for the E6-mediated degradation of p53. It was shown that E6 and E6AP function as an E3 ligase complex to induce ubiquitination of p53 (106, 191). Besides its role in the degradation of p53, it has been shown that mutations in the gene encoding E6AP (*UBE3A*) are the cause of Angelman syndrome, a severe neurological disease (34, 189), as will be discussed in the final section of this chapter. Subsequent bioinformatic examination revealed many proteins with high similarity to the C-terminal 350 amino acids of E6AP (104) and studies have shown that HECT E3s play a broad spectrum of roles in many biological processes from yeast to human, making them one of the two major classes of E3 ligases. HECT E3s are mechanistically distinct from RING E3s in that they participate directly in the chemistry of protein ubiquitination by forming ubiquitin-thioester intermediates (193). HECT E3s appear to be modular proteins, with the N-terminal part of the proteins serving as a substrate recognition domain, while the C-terminal HECT domains function as the catalytic domain. The HECT domain contains all the determinants required for accepting ubiquitin from the E2 and forming a ubiquitin-thioester intermediate (104). The crystal structure of the HECT domain of E6AP showed that the HECT domain adopts a bilobal structure, with an elongated N-lobe connected to a globular C-lobe via a short hinge loop (Illustration 1.1) (102). The E2 binding site and the active site cysteine residue (Cys^{820}) are located in the N-lobe and the C-lobe, respectively, together constituting the catalytic core of the HECT

domain by forming a U-shaped pocket. The Cys^{820} residue is located in a four-residue active site loop, which is positioned at the interface between the N-lobe and the C-lobe (102). The active-site loop is the most highly conserved region among the HECT domains, and it was shown that mutations in any of these four residues resulted in significant reduction in the formation of ubiquitin-thioster bond, suggesting that the contacts made by the active-site loop between the N and the C lobe is important for the catalytic activity. Furthermore, mutations of N-lobe hydrophobic residues surrounding the active-site loop reduced ubiquitin-thioester formation by more than 90%, suggesting that the N-lobe portion in the cleft is also critical for the activity. The transfer of ubiquitin from E2 to E3 requires nucleophile attack on the E2-ubiquitin thioester by the active site cysteine of the HECT E3. However, the crystal structure showed that the two active-site cysteines of E6AP and UbcH7 are separated by 41Å. Given the surprisingly large distance between the two active site cysteines, a major conformation shift between the two lobes appears to be necessary for the trans-thiolation reaction between the E2 and the HECT domain to occur (102). It is conceivable that the binding of a ubiquitin-conjugated E2 on the HECT domain might trigger a change in the relative orientation between the N-lobe and the C-lobe to bring the distance between the two cysteine residues closer. The crystal structure of the HECT domain from another HECT E3, WWP1, showed that the HECT domain of WWP1 closely resembles that of the E6AP HECT (222). Interestingly, however, the relative orientation between the N-lobe and the C-lobe of the WWP1 HECT differed significantly from that of the E6AP HECT, due to the rotation around the hinge loop connecting the two lobes. This resulted in

positioning of the C-lobe closer to the E2 binding site within the N-lobe, with the distance between the active site cysteine of the WWP1 HECT domain and that of a modeled E2 shortened to be 16Å. This differential conformation of the HECT domains appears to be possible due, at least in part, to the flexibility of the hinge region between the the N and C-lobes, as the mutations within the region abrogated the ubiquitin-thioster bond formation. An attractive model explaining the necessity for flexibility within the HECT domain might be that, for each cycle of the ubiquitin transfer, the C-lobe receives ubiquitin from E2 at one side of the HECT domain and transfers it to the substrate bound to the N-terminus, which is at the opposite side (222, 252). Importantly, this mechanism suggests that the interface between the N and C-lobes need to be rearranged. More studies are needed to better understand the structure and function of the HECT domain. Recently, the crystal structure was solved for the HECT domain of Smurf2 (169), the HECT E3 involved in TGF-β signaling pathway (201). The structure showed a similar overall L-shape of the N- and C-lobe, except that the distance between the two active site cysteines is predicted to be even farther than that of E6AP/UbcH7 pair (Illustration 1.1).

A.

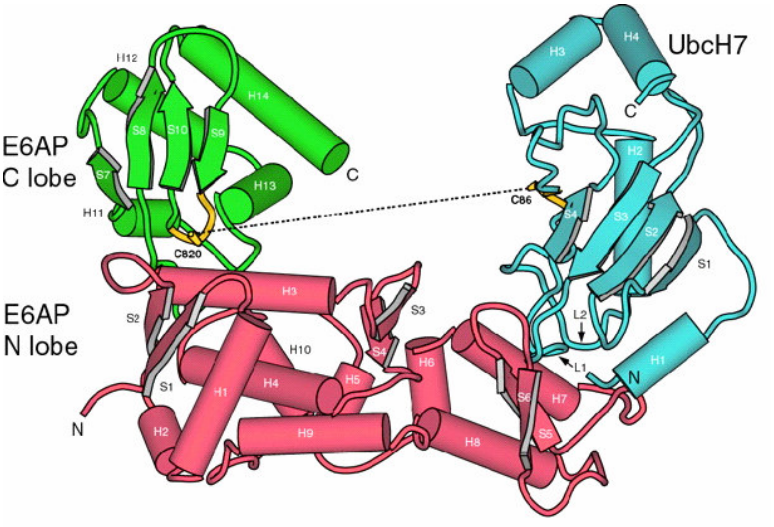
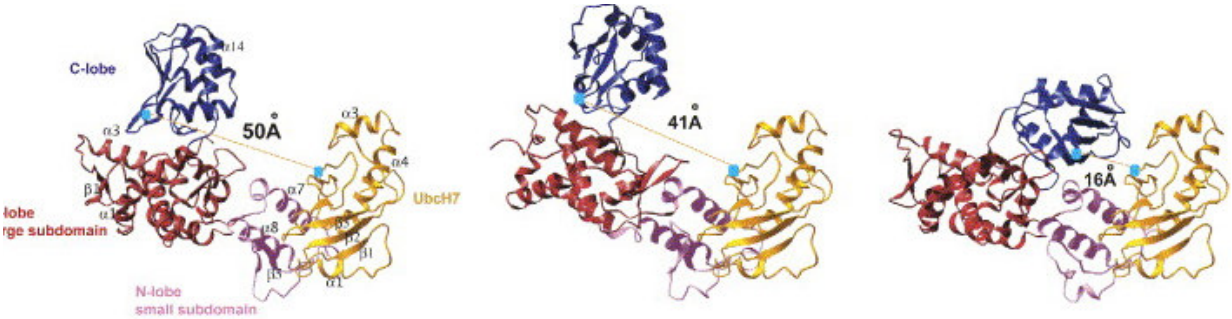


Illustration 1.1 The crystal structures of three HECT E3s. A. The detailed view of the E6AP HECT domain/UbcH7 complex. E6AP hect domain N lobe, C lobe, and UbcH7 are colored in green, red, and cyan, respectively. The two active-site loops containing Cys residues are colored yellow. The dotted line indicates distance (41Å) between the active-site cysteines of E6AP and UbcH7. Adapted from Huang et al, 1999

B.



Smurf2 & modeled UbcH7 E6AP & UbcH7 WWP1 & modeled UbcH7

Illustration 1.1 continued. The crystal structures of three HECT E3s. B. Comparison of the overall structure of three different HECT E3s. Smurf2 HECT domain revealed the most open L-shaped conformation between the N-lobe (red and pink) and the C-lobe (blue), and the WWP1 HECT domain showed the closest gap between the two active site cysteines. Reprinted from Ogunjimi et al (169). Copyright (2005), with permission from Elsevier.

RING E3

The RING E3s are the largest known class of E3 ligases. The RING finger was first described as a functional module that mediates protein-protein and protein-DNA interaction (146). The RING domain is typified by eight cysteine and one histidine residues that constitute a zinc binding pocket (44). Unlike the HECT E3s, RING E3s are thought to promote ubiquitination of substrates by serving as bridging proteins between the E2s and substrates, facilitating the direct transfer of ubiquitin from E2s to lysine residues of substrates. A comparison of the crystal structure between the c-Cbl RING domain and the E6AP HECT domain bound to the same E2, UbcH7, indicated that a common motif in the E2 is recognized by the two different types of E3s (253). The RING domain ligases can be divided into two classes, either single polypeptide E3s (*e.g.* Mdm2) or multi-enzyme complexes, namely the Cullin-RING ligases (CRLs) superfamily (173). The archetypical CRL is the SCF (Skp1/Cdc53/Cul1/F-box) E3 complex. The SCF ligase consists of Rbx1 (also known as Roc1 or Htr1), Skp1, Cul1, and a F-box protein (148, 173). Cullins are a family of proteins that are characterized by the presence of a distinct globular C-terminal domain (cullin homology domain), with which a RING domain protein Rbx1 associates, and an N-terminal domain recruits substrates by binding to Skp1 and various adaptor proteins (173). The key feature of CRLs is that each cullin can associate with many different substrate adaptor proteins, forming distinct E3 ligase complexes that share common catalytic core components yet recognize different substrates. The substrate adaptor proteins include F-box proteins, BTB (broad complex, tramtrack, bric-a-brac) domain proteins, and SOCS/BC (suppressor of cytokine

signaling/elongin-BC) box proteins (115, 241). These different adaptor proteins can bind to different cullins, although F-box proteins require Skp1 to mediate the interaction with cullins (8). Due to the great diversity of substrate adapter subunits, it is possible to imagine that there might be more than a hundred of distinct CRLs with different substrate selectivity in eukaryotic cells.

U-box E3

The U-box containing proteins are generally considered to be the third type of E3 ligases. The U-box is a highly conserved ~70 amino acid domain whose structure is closely related to the RING domain, despite the lack of the hallmark zinc-binding residues of the RING finger (82). The best characterized U-box E3 ligase is yeast Ufd2, one of the five gene products (Ufd1-5) isolated in a genetic screen (111). The screen was designed to isolate mutants in the proteolytic system that disrupts degradation of an artificial ubiquitin fusion substrate, ubiquitin-ß-galactosidase (UFD, ubiquitin fusion degradation, substrate). Later, it was shown that Ufd2 functions in a non-canonical manner, by elongating polyubiquitin chains that were pre-synthesized by E1, E2, and an E3 (Ufd4) (125). Ufd2 was able to catalyze only extension of short oligo-ubiquitin chains on the artificial substrate, which were catalyzed by Ufd4, a HECT E3 ligase. Because of this distinct activity, Ufd2 was designated as "E4" (98, 125). A database search revealed many proteins with the conserved U-box domain and all of them have been shown to possess E3 ligase activities (83). It is not known whether all U-box containing E3s function as E4.

PHD domain E3

Recent reports have shown that PHD (plant homeodomain) domain proteins can function as E3 ligases (36). PHD domains typically exhibit a C4HC3 (four cysteine, one histidine, three cysteine) signature and bind to zinc ions, with characteristics similar to the RING domain (1). In spite of the similarity in the core region, a structural analysis has revealed the differences of overall surface areas between PHD domains and RING domains, particularly the E2 binding regions (46). A distinct feature of PHD domains is that most of the PHD domain proteins identified are nuclear proteins (36) and many PHD domain proteins have been shown to bind to chromosomes (11). Consistent with the observations, the PHD domain of the nucleosome remodelling factor (NURF), the largest subunit of the ATP-dependent chromatin-remodelling complex, have been shown to mediate a direct association of the complex with the methylated histones (240), further suggesting that the PHD domain functions as chromosomal binding module. Furthermore, the recognition mediated by the PHD domain was shown to be preferential to specific types of methylation (134). However, several viral PHD domain proteins have been shown to function outside of the nucleus (75, 145). Human herpes virus 8 (HHV8) encodes two PHD domain containing viral proteins, K3 and K5, which have been shown to promote immune evasion by ubiquitination and down-regulation of MHC1 (major histocompatibility complex I). Further studies have shown that several cellular PHD domain proteins also function as E3 ligases in the cellular signaling pathways, including FANCL in the DNA repair pathway, MITOL in the regulation of mitochondrial dynamics, and MEKK in the MAP

kinase pathway (79, 147, 246). Despite the fact that several PHD domain proteins have been demonstrated as E3s, it is still not clear whether all PHD domains function as E3 ligases.

E4 activities

As mentioned above, Ufd2 has been shown to possess a specialized type of activity, which is distinct from other E3 ligases. This type of activity seems to exist in other cases besides the UFD pathway, leading to a proposal that the E4 activities represent a novel distinct class of ubiquitinating enzyme (98). A well characterized transcriptional cofactor, p300, has been shown to be required for the extension of ubiquitin chains on p53, which is pre-monoubiquitinated by Mdm2, leading to its degradation (78). Interestingly, p300 alone cannot catalyze polyubiquitination of p53, similar to Ufd2, further suggesting that p300 indeed possesses the so-called E4 activity. No domains similar to known E3 motifs, such as HECT or RING domain, are present in p300. CHIP (C-terminus of Hsc70-interacting protein) is another example of E4 activity. CHIP is a U-box containing protein with an apparent E3 ligase activity, and it has been shown to be capable of enhancing the ubiquitinating activity of Parkin on its substrate Pael receptor (Pael-R). (109). Bul1 and Bul2, two yeast proteins that bind to Rsp5 HECT E3 ligase, were proposed to possess E4 activities (86). It has been shown that in the absence of Bul1 and Bul2, Rsp5 monoubiquitinates Gap1 general amino acid permease to deliver it to the plasmamembrane in a nitrogen-deplete condition, wherease Gap1 is polyubiquitinated when Bul1 or Bul2 is overexpressed. However, further

biochemical demonstration is needed to prove that Bul1 and Bul2 have such chain-extending activities, as they do not contain apparent E3 ligase activities.

As a summary, illustration 1.2 describes the complex hierarchy of the E1-E2-E3 ubiquitinating enzymes. A growing number of reports show that additional components or variants are involved in the ubiquitin pathway besides the E1-E2-E3 core ubiquitinating enzymes, adding much more complexity to the pathway. Some of the components discovered so far are discussed below.

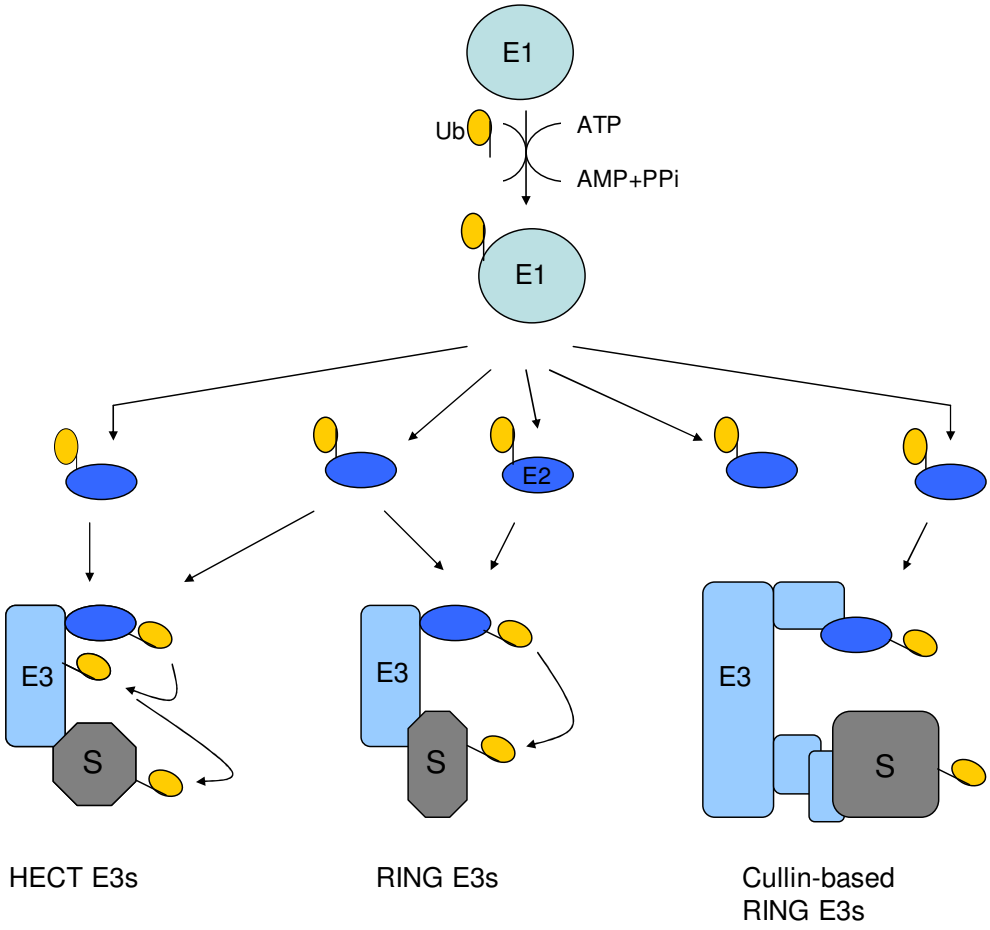


Illustration 1.2 Diagram for the enzymatic cascade of ubiquitination. Cells contain one or few E1, a dozen of E2s, and numerous E3s. E3 ubiquitin ligases are responsible for the specificity of attachment of Ub to the target protein through the recruitment of both an E2 thiolester and a specific substrate. More than one E2 can work with a given E3 and several E3s can use a single E2. HECT E3s are different from RING E3s in that they form intermedieate thioester bonds with ubiquitin on their active-site cysteine.

1.3 Deubiquitinating enzymes

Like other types of protein modifications (*e.g.*, phosphorylation), ubiquitination is a reversible process, as attachment of ubiquitin to target proteins can be reversed by a group of enzymes called deubiquitinating enzymes (DUBs) (4). During the past few years, DUBs have emerged as important regulators of many physiological processes. It is currently estimated that there are as many as 90 DUBs in human cells (101), making them the second largest class of enzymes in the ubiquitin system, next to the E3 ligases. DUBs catalyze cleavage of the isopeptide bond between the C-terminus glycine residue of ubiquitin and a lysine residue of the conjugates. Another important role of DUBs is to generate a mature form of ubiquitin from precursors, which otherwise are incompetent to be conjugated to substrates, as discussed below.

Functions of DUBs

Because ubiquitination of proteins often leads to their degradation by the proteasome, DUBs can affect the stability of target proteins by removing ubiquitination of target proteins. The known biochemical functions of DUBs are discussed here.

1) Processing of ubiquitin precursors

In all eukaryotes, ubiquitins are initially produced as C-terminally extended precursors (4). They are either fused to ribosomal subunits or to ubiquitins as multimers that also have additional amino acid extensions following the last ubiquitin monomers (62). Although it is not clear why ubiquitin is produced as a precursor form, it might be to ensure high-level expression, as the ribosomal proteins to which they are fused are highly abundant proteins. Ubiquitin, itself, does not need to be expressed in a precursor

form in order to be conjugated to cellular proteins. The processing function seems to be redundant among DUBs, as any single deletion of DUBs in yeast is non-lethal, except for Rpn11 which seems to be an essential enzyme for a different reason (discussed below). Many DUBs have shown to be competent for this activity *in vitro* (4).

2) Regenerating free ubiquitin monomers.

One of the most important roles of DUBs is to rescue ubiquitin molecules prior to either proteasomal or lysosomal degradation of conjugated substrates to regenerate a free pool of ubiquitins. This role of DUBs seems to be important for cells to carry out many biological processes, as mutations of the DUBs required for recycling of ubiquitin result in growth defects in many different conditions (6, 133, 215). Ubp4/Doa4 of *S. cerevisae* is a membrane-associated DUB which has shown to function at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins *en route* to the vacuole (215). It has been shown that in *ubp4* mutant cells, decreased viability was observed in correlation with decreased free ubiquitin and defective endocytosis of receptor proteins. In agreement with its primary role, phenotypes of the *ubp4* mutant can be rescued by overexpressing free ubiquitin. Interestingly, increased levels of polyubiquitinated membrane cargo proteins were detected in the *ubp4* mutants, suggesting that Ubp4-mediated deubiquitination of endosomal cargos is a prerequisite for their degradation in the vacuole (52). Ubp6 (Usp14 in mammals) is a proteasome-associated DUB which has been suggested to rescue ubiquitin from proteosomal degradation (133). The *ubp6* mutant cells exhibit a severe growth defect under many conditions, which can be rescued by overexpressing

ubiquitin, thereby suggesting that the phenotypes are due to depletion of free ubiquitin (27).

3) Reversing substrate ubiquitination

Degradation of ubiquitinated proteins by the 26S proteasome obviously is an irreversible process. The dynamic regulation of substrate ubiquitination and deubiquitination seems to be important for many substrates. I describe several examples where DUBs function as critical regulators of given pathways.

CYLD (Cylindromatosis), the first DUB linked to a human genetic disorder, familial cylindromatosis (12), was shown to have a tumor-suppressive role by inactivating the NF-kB pathway (17, 127, 220). TRAF2 and TRAF6 (TNFR-associated factor 2 and 6, respectively), the heterodimeric adapter proteins that bridge the interaction between TNF receptors and the downstream proteins, possess the RING domains and ubiquitinate multiple substrates in the pathway, including themselves (26). It was shown that CYLD, by associating with the E3 complex, deubiquitinates multiple targets in the complex including the auto-ubiquitinated E3s, thereby inactivating the NFkB pathway. It appears that a loss of CYLD activity in the deubiquitination of the targets in the NFkB pathway is the underlying mechanism for the cylindromatosis. More recently, CYLD was also shown to deubiquitinate Bcl-3, thereby preventing its translocation into the nucleus and stimulation of NF-kB mediated-transcriptional activation (153). These data suggests that CYLD inhibits different steps of the NF-kB pathway by deubiquitinating multiple substrates within the pathway.

FANCD2, the key mediator protein in the FA (Fanconi Anemia) pathway, is monoubiquitinated upon DNA-damage and localizes into the nucleus to facilitate DNA repair (38). It was found that USP1 (ubiquitin specific protease 1) is a critical regulator of the FA pathway (167). It was shown that deubiquitination of FANCD2 by USP1 is required for the recycling of the nuclear protein back to the cytoplasm, which inhibits further activation of DNA repair. Conversely, persistent monoubiquitination of FANCD2 in the USP1-depeleted cell is likely to result in hyper-activation of the FA pathway and chromosomal aberrations. USP1 was also shown to deubiquitinate PCNA (proliferating cell nuclear antigen), monoubiquitination of which is required to safeguard against error-prone translesion DNA synthesis (103).

Regulation of p53 activity is achieved by many types of modifications, including ubiquitination. It was shown that Mdm2-mediated ubiquitination of p53, which leads to destabilization of the protein, can be reversed by a deubiquitinating enzyme HAUSP (herpesvirus-associated ubiquitin specific protease; also known as USP7), thereby leading to stabilization of p53 (137). However, a more recent study has shown the opposite result, that stabilization and activation of p53 were observed when HAUSP was disrupted in human cells (37). It was also shown that HAUSP deubiquitinates Mdm2, which results in stabilization and activation Mdm2, that, in turn, leads to the destabilization of p53. The pathway seems to be far more complicated than once thought, with the contribution of several other factors have also been shown in the multi-faceted pathway (16, 159).

Taken together, it is evident that the deubiquitination of proteins by DUBs is a crucial mechanism for regulating many pathways, and it is likely that DUBs are widely used in other processes as well. It is interesting to note that CYLD, USP1, and HAUSP all have multiple substrates in their repsective pathways to deal more effectively with the corresponding signals. Significantly, activities of many DUBs have shown to be strictly regulated by different signaling events (85, 103, 108, 159).

4) Disassembly of free polyubiquitin chains

Accumulation of excessive free polyubiquitin chains in the cells can inhibit proteosomal degradation and eventually become toxic to cells (3). This can be due either to limited available ubiquitin monomers or competitive inhibition of binding of polyubiquitinated substrates to the proteasome (3). These free unanchored chains are likely to be the byproducts of proteosomeal degradation, as the polyubiquitin chains must be removed from substrates and cleaved into monomers for recycling, prior to substrate degradation. Cells seem to have developed specialized DUBs to cope with this potential problem, as Ubp14 in *S. cerevisae* (Isopeptidase T in mammalian cells) has been shown to disassemble, preferentially, the bulk of unanchored free chains (3). Interestingly, Ubp14 has been shown to remove ubiquitins from the proximal end of the chain (the ubiquitin with free glycine carboxyl group) and has also been shown to have marked preference to unanchored free chains *in vitro*, consistent with its demonstrated role in the proteasome pathway. Although yeast cells deficient in Ubp14 display increased free polyubiquitin chains and inhibition of proteosomal degradation of substrates, they seem to be viable under normal conditions, although *ubp14*Δ cells are

highly sensitive to canavanine. However, its orthologs in Dictyostelium and Arabidopsis, UbpA and AtUBP14, respectively, which can functionally replace yeast Ubp14, were shown to be essential during the early development of each organism (47, 141). However, cells with deficient Ubp14 inhibited proteasomal degradation of only selective substrates, suggesting that redundant DUBs exist for free chain disassembly in the proteasomal degradation pathway (54), consistent with the fact that the *ubp14*Δ cells are viable.

Classes of DUBs

There are at least five subgroups of DUBs characterized to date, based on their sequence similarities and biochemical activity. Four of the five groups of DUBs are cysteine proteases and one group contains metal-dependent proteases.

1) UCHs and UBPs

Ubiquitin C-terminal hydrolases (UCHs) and ubiquitin specific proteases (UBPs) are two major groups of DUBs. UCHs are generally small proteins (20-30kDa) that preferentially cleave ubiquitins from small adducts such as ubiquitin precursors (4). Although UCHs were the first known DUBs, their physiologic functions and substrate specificities are poorly understood. The best characterized UCH member is UCH-L1, the neuron-specific protease. UCH-L1 has been presumed to be critical for cytoplasmic protein degradation and ubiquitin recycling and has been linked to Parkinson's disease (142). Recently, it was found that the level of UCH-L1 was low in AD (Alzheimer disease) model mice and UCH-L1 was shown to be required for normal synaptic function and cognition function (74).

Compared to UCHs, UBPs are much larger in size (60-300kDa) and seem to have a wider range of substrate specificity. It is estimated that more than 80% of total DUBs are UBPs (30). In *S. cerevisiae*, 16 of 18 DUBs characterized are UBPs, including Ubp2, which will be discussed in detail in this dissertation. The rest of the DUBs in budding yeast include a UCH family protease and a JAMM motif protease. UBP members do not exhibit high sequence homology, unlike relatively well conserved UCHs. The only conserved regions detected are the two short stretches of highly conserved sequences, termed the Cys box, which generally comprises of up to 19 amino acids that contain the highly conserved "GxTCY" signature, and the His box, which generally contains 60-90 amino acids with a conserved "GHY" signature (4, 101). Together with the conserved Asp residue, these domains form a "catalytic triad"(4). The crystal structures of representative members of both UCHs and UBPs have been solved for both free enzymes and the enzmymes in a complex with ubiquitin (101, 112). In order to isolate stable complexes, both studies used ubiquitin aldehyde (Ubal), which forms a relatively stable hemithioacetal intermediate with the active-site cysteine (88, 101). These studies revealed several interesting common features between UCHs and UBPs. First, the structures of the catalytic core triad are closely related to each other and are superimposable to that of other cysteine protease such as papain (4, 101, 112). Secondly, the conformation of the catalytic cores are switched to active forms, in which the the catalytic Cys, His, and Asp residues are close together, only when the Ubal was complexed, suggesting that they undergo a conformational change upon binding to ubiquitin. Together with the fact that the sequences that form this catalytic triad have

been shown to be highly conserved with other representative UCHs and UBPs, the structure data strongly suggest that mechanisms of proteolysis among UBPs are quite similar. The rest of the sequences are highly divergent, generally containing long extended sequences, whose functions are largely uncharacterized, although it is assumed that those non-conserved sequences might be responsible for determining substrate specificities. Consistent with this, the N-terminus divergent domain of HAUSP has been shown to interact with its substrate, p53 (101).

2) OTU proteases

OTU (ovarian tumor)-related proteases are a recently discovered family of cysteine proteases, initially predicted in a bioinformatic study that found a set of proteins that contain sequences similar to other cysteine proteases (150). It was suggested that the predicted structures of these proteases are unrelated to previously known proteases. Based on sequence analysis, it was suggested that conserved sequences surrounding cysteine and histidine comprise a catalytic dyad which is indispensable for the biochemical activities, similar to the UBP family proteases. Subsequent reports have shown that the OTU family proteases are indeed ubiquitin proteases. Cezanne (cellular zinc finger anti-NF-kB) was the first described OTU protease with deubiquitinating activity, shown by *in vitro* cleavage of ubiquitin monomers from synthetic chains and substrates (60). The best characterized OTU domain-containing protein is A20, a potent negative regulator of the NF-kB pathway. Interestingly, it was found that A20 contains two opposite ubiquitin-editing activities, the N-terminal OTU deubiquitinating domain and the C-terminal zinc-finger sequences

with E3 ligase activity (232). It has been shown that the sequential deubiquitination and ubiquitination of its substrate RIP (receptor interacting protein) by A20 is required for inhibiting downstream events in the NF-kB pathway. Otubain 1 and Otubain 2, two OTU domain proteases recently identified using Ubal as a trap, have been shown to exhibit deubiquitinating activity against Ub-GFP fusion *in vitro* (9).

3) Josephin domain proteases

The Josephin domain, named after Machado-Joseph (MJ) disease, is a conserved monomeric domain which folds into a globular conformation and possesses deubiquitinating activity (29). A bioinformatic study has revealed that the Josephin domain is present in at least 30 predicted proteins (151). The best characterized Josephin domain containing protein is Ataxin-3. Ataxin-3 belongs to the family of polyglutamine proteins, which are associated with nine different neurodegenerative disorders, and it contains the N-terminal Josephin domain followed by two ubiquitin interacting motifs (UIM) domain, poly-Q stretch, and a short variable tail (151). The poly-Q stretch has been shown to to mediate the interaction with VCP/p97 , an ATPase involved in protein quality controls in ER (93, 154), suggesting a role of Ataxin-3 in protein quality control. The NMR structure of Ataxin-3 has demonstrated conserved characteristics of other cysteine proteases which are represented by the catalytic triad that includes cysteine, histidine, and aspartate. The chemical shift data have also revealed that the Josephin domain can bind to ubiquitin by itself. The UIM domains are thought to be linked to the DUB activity and to recruit polyubiquitin chains for orienting the substrates for efficient cleavage. Thus, it has been proposed that Ataxin-3 functions as a polyubiquitin chain-

editing protease (151) and, recently, the deubiquitinating activity of Ataxin-3 was shown to facilitate degradation of misfolded ERAD (ER-associated degradation) substrates, possibly by editing the chain length of the substrates (228).

4) JAMM motif proteases

JAMM (JAB1/MPN/Mov34 metalloenzyme) motif proteases are distinct from the rest of the DUBs in that they are zinc-dependent metalloproteases, not cysteine proteases. They share a distinct conserved, putative metal-binding motif, called the JAMM motif (2). The metalloprotease activity depends on absolutely conserved His and Asp residues, both of which constitute a zinc-binding pocket (4). The best characterized enzymes that fall into this group are Rpn11/POH1 and Csn5, which are intrinsic components of proteasome and COP9 signalosome (CSN), respectively (4). The lid of 19S proteasome and CSN are closely related complexes, with Rpn11 and Csn5 as the subunits that are most closely related to the two complexes (2). The JAMM motif of Rpn11 seems to be essential for its activity, as mutation in the motif stabilized ubiquitinated substrates and rendered yeast cells lethal (223). Since Rpn11 was found to be associated with the proteasome, it has been suggested that the deubiquitination step by Rpn11 is essential for proteasomal degradation of substrates. CSN, the multi-functional complex that cleaves Nedd8 from cullin, is required for activation of the cullin-based SCF E3 ligase complex. It was found that the JAMM motif in the Csn5 subunit is the underlying Nedd8-cleaving activity of CSN (35). More JAMM motif proteases have been found, such as AMSH (associate molecule with SH3 domain of STAM), whose protease activity is involved in a negative regulation of receptor endocytosis (157).

1.4 Ubiquitin binding domains

One of the most important questions in the ubiquitin field that remains largely unanswered is how ubiquitination is transmitted to the downstream events. Owing to the past few years of extensive studies on the accessory components in the ubiquitin pathways such as ubiquitin binding domains (UBDs), we are beginning to unravel the mysteries of ubiquitin-mediated signal transduction.

UBDs are independently folded modular domains that non-covalently bind to ubiquitin (92). The first ubiquitin binding site to be characterized was a subunit in the 26S proteasome, S5a (Rpn10 in yeast) (45, 247). The 30 amino acids in the C-terminus of the protein, now known as the ubiquitin interacting motif (UIM), were shown to be sufficient and necessary for binding to a hydrophobic surface of ubiquitin (247). Significantly, it was shown that the S5a subunit displays selective affinity towards polyubiquitin chain, as opposed to monoubiquitin, consistent with its role in the recognition of polyubiquitinated proteasomal substrates. The information from this work was used to perform subsequent bioinformatic studies and revealed several UIM-containing proteins in the proteosomal and lysosomal systems (96, 176). The Ubiquitin-associated domain (UBA) was first identified in another bioinformatic study (95) and was later found to be capable of binding to ubiquitin as well (233), representing the second UBD to be characterized. The latter work first described that Rad23 and Dsk2, two major proteins involved in delivering K48-linked polyubiquitin chains to proteasome, contain UBA domains. Other UBDs have been characterized over the past

few years, some of them by using proteomics approaches that have led to identification of the CUE (coupling ubiquitin to endoplasmic reticulum degradation) (204) domain, the GAT (gga and tom1) (203) domain, the PAZ (polyubiquitin-associated zinc finger) domain (198), the VHS (vps27, hrs, sTAM) domain (162), the NZF (npl4 zinc finger) domain (160), the GLUE (gRAM-like ubiquitin-binding in eap45) domain (205), and the UEV (ubiquitin conjugating enzyme variant) domain (70).

Although the presence of UBDs suggests that the proteins may function in the ubiquitin binding, functional relevance of the proteins in the given pathways remains largely uncharacterized. So far only few roles of the UBD-containing proteins are ascribed to the UBDs in *S. cerevisae*, and most seem to have roles in recognizing endocytic cargo and polyubiquitinated substrates in proteasomal pathway. The fact that some of the deubiquitinating enzymes (*e.g*. Ubp14), a RNA helicase (YHR419W), and a metabolic enzyme (Gts1) also contain UBDs (92) suggests that UBDs might have broader roles outside the endocytic and proteasomal pathways. UBDs are often found in combination with various other domains, further suggesting that ubiquitin binding is involved in many processes.

The best characterized UBD-containing protein is yeast Rad23 (mammalian hHR23A). Rad23 contains an N-terminal UBL (ubiquitin-like) domain and two UBA domains in the middle and C-terminus of the protein, and Dsk2 has an N-terminal UBL domain and C-terminal single UBA domain. Rad23 was originally characterized for its role in nucleotide excision repair (161). Recently, however, it was shown that the UBA domains of Rad23 have an important role in binding to polyubiquitinated proteins (24).

Furthermore, the ERAD (ER-associated protein degradation)-associated E3 ligase Ufd2 and the Rpn1 proteasomal subunit compete for binding to the UBL domain of Rad23, suggesting that Rad23 is a shuttling protein that couples between substrate ubiquitination and delivery to the proteasome by having two different UBDs (56, 124). Importantly, yeast cells lacking UBA domains of both Rad23 and Dsk2 are defective in proteolysis of UFD substrates, suggesting that they cooperate, perhaps redundantly, to recognize a subset of the polyubiquitinated chains and deliver them for proteasomal degradation (179). Also, a genetic screen identified Rad23 and Dsk2 as essential proteins for ERAD (158), further suggesting that they are widely used essential shuttling proteins for delivery of polyubiquitinated proteins.

Related to the fact that Rad23 specifically recognizes polyubiquitinated proteins destined for proteosomal degradation, an important question is how UBA-domains recognize different forms of ubiquitin chains, such as K48 or K63-linked polyubiquitins or monoubiquitin. The systematic analysis of the polyubiquitin chain-interaction properties of 30 UBA domains showed that some UBA domains display linkage-selective polyubiquitin binding and some domains bind different chains, and monoubiquitin, in a nondiscriminatory manner (178). For example, the second UBA domain of Rad23 was shown to bind to K48-linked chains in preference to K63-linkages, consistent with Rad23's demonstrated role as a shuttle protein in the proteasomal degradation. However, some of the UBA domains that have been shown to be involved in proteasomal degradation have demonstrated non-selective preference to different chain types, raising the possibility that the full-length proteins might behave

differently than their individual UBA domains due to the presence of other domains in the proteins. In general, there is a wide range of affinities between UBDs and their substrates, although the affinities are shown to be very low, with typical Kds ranging from 10 to 500uM (92). The inherent low affinities of the UBDs to ubiquitin may reflect the dynamic nature of the physiologic interactions that enable the ordered transfer of ubiquitinated substrates to acceptors.

1.5 Polyubiquitin chain types

Ubiquitin has seven internal lysines (K6, K11, K27, K29, K33, K48, and K63) and all have shown to be capable of accepting ubiquitin molecules to form polyubiquitin chains in cells, as demonstrated by mass spectrometry analyses of purified ubiquitin conjugates from cells (171, 216). Recently, types of polyubiquitin chain linkages have become an important topic in the ubiquitin field due, in part, to increasing evidence that non-proteolytic linkages (*e.g.* K63-linkages) play important roles in physiological processes. The consequence of polyubiquitination depends on which lysine was used for synthesizing the polymer. K48-linkages are considered to be the major type of polyubiquitin chain and lead to degradation of the conjugates by proteasome (21). Consistent with its essential role in proteasomal degradation, K48 has been shown to be the only lysine residue in ubiquitin essential for the viability of yeast cells, although mutations of other lysines moderately affect cell growth (208).

K63-linkages are probably the second most abundant chain type in cells and have been implicated in several processes, with the best characterized role being in

DNA repair (97, 208). The underlying basis for the role of K63-linkages in DNA repair has been shown to involve PCNA (proliferating cell nuclear antigen), a DNA-polymerase sliding clamp involved in DNA synthesis and repair, and it has been shown that K63-linked polyubiquitination of PCNA is required for error-free DNA replication upon DNA damage (94), by blocking the activity of error-prone TLS (translesion synthesis) polymerases (28). Furthermore, cells deficient in K63-linkages have been shown to be hypersensitive to UV-induced mutation and DNA-crosslinking agents (28), further suggesting that the K63-linkages play critical roles in maintaining the genomic integrity. The K63-linked polyubiquitination of PCNA was found to be catalyzed by an E2 heterodimer complex that consists of Ubc13 and Mms2, a yeast E2 variant (UEV; homologs of E2s that lack active site Cys) (97). The question as to how K48 or K63-linkages are selectively assembled by specific enzymes remains one of the important challenges in the field. A recent structural analysis of Mms2/Ubc13 complex suggested that Mms2 might have a role in allowing selective accession of K63 of donor ubiquitin into the active site pocket of Ubc13, promoting nucleophile attack of acceptor thioester bond selectively by the K63 residue (53).

Another well characterized pathway that involves K63-linked polyubiquitination is NF-kB signaling pathway (43, 116). During the NF-kB activation, proteasome-dependent IkBα degradation is triggered by IKK (IKß kinase) complex-mediated phosphorylation and ubiquitination of IkBα (197). How IKK is activated had been mysterious until the discovery that IKK activation is dependent on K63-linked polyubiquitination on several key regulators in the pathway (43). It was shown that

upon cellular stimulation, TRAF2 and TRAF6, heterodimeric RING E3s in the pathway, undergo K63-linked autoubiquitination and ubiquitinate other targets. Instead of inducing proteasomal degradation, the K63-polyubiquitinations lead to the enhanced phosphorylation of IkBα, which is indicative of the activated IKK. One of the initial targets is an adaptor protein RIP (receptor interacting protein), and the modified RIP recruits NEMO, a regulatory subunit of the IKK complex, and TAK kinase complex. Subsequently, the recruited IKK complex is activated by TAK kinase-mediated phosphorylation (225), which in turn phosphorylates IkBα. Recently, it was shown that NEMO displays strong preferential binding to K63-linkages over K48-linkages, and a defective binding of NEMO to the polyubiquitinated RIP results in the proteasome-dependent degradation of RIP (238). Interestingly, it was shown that the K63-polyubiquitinated RIP is destabilized by A20, a dual function protein with a DUB and a E3 activities, which deubiquitinates the K63-linkages and subsequently ubiquitinates via K48-linkages (232). Conceivably, the NEMO binding of polyubiquitinated RIP might protect RIP from being targeted by A20. Notably, K63-linked polyubiquitination in the NF-kB pathway is also dependent on the Ubc13/Uev1A E2 complex (Mms2 in yeast), suggesting that the Ubc13/Uev1A is involved in more than one pathway involving K63-linkages.

Other processes that involve K63-linked polyubiqiuitination include Rsp5-mediated receptor endocytosis (66, 206), Rsp5-mediated mitochondrial inheritance (64), Rsp5-mediated cell wall biogenesis (121), and a potential regulation of ribosomal function (by an unknown conjugating enzyme) (207). Although it has been shown that

K63-linked polyubiquitination of certain substrates lead to proteasomal degradation *in vitro* (187), whether or not that reflects what naturally happens in the cells remains to be determined. The solution structures of K63-linked di-ubiquitin have been shown to be significantly different from that of K48-linked di-ubiquitin (Illustration 1.3). The K48-linked di-ubiquitin adopts a closed conformation with possible intra-molecular interactions between the surface hydrophobic residues (L8, V70, I44) of ubiquitin whereas the K63-linked di-ubiquitin adopts an extended conformation with no intra-molecular contacts. This apparent structural difference between the two types of chains is likely to be the basis for performing distinct functions (175).

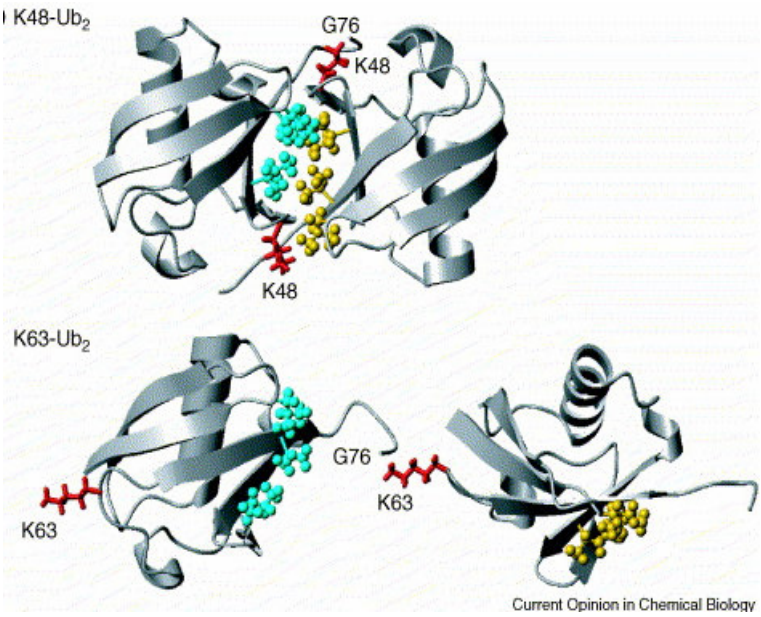


Illustration 1.3 Solution structures (NMR) of K48-linked di-ubiquitin (top) and K63-linked di- ubiquitin (bottom). The lysine residues are shown red and the surface hydrophobic residues (L8, V70, I44) are shown in cyan and gold. Reprinted from Pickart and Fushman (175). Copyright (2004), with permission from Elsevier.

Several enzymes have been shown to be capable of catalyzing K29-linked chains *in vitro*, including two HECT E3s Ufd4 (188) and KIAA10 (229), although the biological relevance of the polyubiquitination still remains elusive. A potential link between K29-linkages and receptor endocytosis was suggested in a recent report (19). This work showed that Itch, a mammalian homologue of Rsp5, catalyzes K29-linked polyubiquitination of Deltex, a positive regulator of the Notch signaling pathway, thereby facilitating the down-regulation of the substrate via endocytosis.

Other types of chain linkage remain largely uncharacterized, including K6-linked chains, whose only biological implication was assigned in the BRCA1/BARD1 E3 complex-mediated autoubiquitination (237). The biological relevance of the K6-linkages and substrates are unknown.

1.6 Functions of Rsp5

Rsp5 is an essential HECT E3 ligase in *S. cerevisiae* that belongs to the Nedd4 (neural precursor cell-expressed developmentally downregulated gene 4) family of HECT E3 proteins. The Nedd4 family proteins share highly related domain organizations and exist from yeast to human (186). Nedd4 family proteins consist of an N-terminal C2 domain, which is a Ca2+/lipid binding domain, two to four WW domains, which have an affinity for proline-rich sequences, and the C-terminal HECT domain. There are multiple Nedd4 members in mammals, Drosophila, *C. elegans*, and *Schizosaccharomyces. pombe*, while there is only one homologue in *S. cerevisae* (201).

Studies over the past decade have shown that Rsp5 is involved in numerous biological pathways and contains multiple substrate specificities. Rsp5 was first implicated in transcriptional regulation, as a temperature sensitive mutant (*rsp5-1*; reverse of spt3 phenotype 5) was isolated to be a suppressor of a *spt3* mutant phenotype (Fred Winston and colleagues, unpublished; cited in (104)). Spt3 is a component of the SAGA (Spt/Ada/Gcn/acetyltransferase) complex which activates transcription of many genes (76). Although direct physical interaction between Rsp5 and the transcription factors have not been reported, the genetic interaction suggested that Rsp5 might have a nuclear function. Other studies have shown that Rsp5 is involved in numerous biological pathways at multiple locations (50, 51, 66, 117, 182, 206, 226). Consistent with this, the mutants of *RSP5* were isolated from multiple genetic screens. So far the well established roles of Rsp5 include transcriptional activation, regulation of endosomal trafficking, RNA export (66, 99, 166). Here I summarize some of the well characterized roles of Rsp5.

The OLE pathway

Although ubiquitination has been implicated in transcriptional regulation in the nucleus by direct ubiquitination of histones or transcriptional machinery (163), it has been shown that Rsp5 mediates transcriptional regulation at the ER membrane (99). This study isolated *OLE1* as a multi-copy suppressor of the cells with deficient Rsp5 activity (expressing catalytically inactive dominant-negative *rsp5* C-A mutant). *OLE1* encodes an essential Δ9 fatty-acid desaturase, a key enzyme synthesizing unsaturated oleic acid. The production of oleic acid is critical for preserving the integrity of

membranes and viability of cells, however, the *ole1*Δ mutant can be rescued by simply supplementing the oleic acid in the growth media (214). It was shown that when the levels of the unsaturated fatty acids in the ER fall, Spt23, the ER-bound transcription factor, becomes ubiquitinated by Rsp5. Strikingly, rather than being degraded by the proteasome, the ubiquitinated Spt23 is specifically cleaved in a proteasome-dependent manner. The processed N-terminal fragment of Spt23, being liberated from the ER membrane, enters the nucleus to induce the Ole1 expression, which catalyzes production of unsaturated fatty acids. How the proteasomal activity is controlled to process Spt23 is unknown, however, this demonstrated that a proteolytic activity of the proteasome can cleave proteins with precision and specitficity. Rsp5 has also been shown to mediate proteasomal activation of Mga2, another ER-bound transcription factor with an overlapping function with Spt23 (99, 200). This OLE pathway represents the minimal essential function of Rsp5 in *S. cerevisiae* at normal growth temperature.

Endosomal trafficking

Studies have shown that the main functions of the Nedd4-family members of HECT E3 ligases are modulation of the activity of membrane receptor proteins (186). Rsp5 has been shown to down-regulate multiple plasmamembrane proteins, including Fur4 (uracil permease) (66), Gap1 (general amino acid permease) (84), and Ste2 (α-factor receptor) (50). Rsp5 has shown to be involved in regulation of both endocytosis of receptor proteins and of biosynthetic cargo such as vacuolar proteases Cps1 (117) and Phm5 (90). The C2 domain is generally thought to recruit Rsp5 to membranes and facilitate the interaction between Rsp5 and its membrane-associated substrates. It has

been shown that the C2 domain is necessary for the down-regulation of Fur4 (226). Additionally, the C2 domain seems to be indispensable for the sorting of biosynthetic vacuolar enzymes (51, 117). Rsp5 has also been shown to be recruited to the biosynthetic compartment by Bsd2, a PPxY-motif containing adapter protein (90), suggesting that Rsp5 is recruited to the membrane substrates via two independent mechanisms. Rsp5 seems to be required for multiple steps in the endocytic pathway, as it has been shown that an *rsp5* mutant is still defective in the internalization of Ste2-ubiquitin fusion, a chimeric receptor protein that does not require ubiquitination (50). Consistent with this observation, the GFP-Rsp5 fusion protein has been shown to be present in multiple locations in the endocytic pathway (226), further suggesting that Rsp5-mediated ubiquitination is required more than at the initial step for receptor internalization. Rsp5 has shown to down-regulate a number of other receptor proteins, including Hxt6 and Hxt7 glucose transporters (128), Zrt1 zinc transporter (72), and Tat1 tryptophan transporter (164). (see Table 1).

The Rsp5-mediated endocytosis of membrane proteins represents one of the well characterized role of the K63-linked polyubiquitination (66, 206). Despite the obvious requirement of K63-linkages in the endocytosis, monoubiquitination appears to be minimally functional in the internalization of Gap1 and Fur4. Consistent with this, the inframe fusion of ubiquitin to Ste2 lacking tail lysine residues have been shown to undergo rapid internalization, suggesting that monoubiquitination is sufficient for the receptor internalization (218). Therefore, it is possible that Rsp5 mediates both types of modification on the membrane proteins.

RNA export

The first link between the ubiquitin system and the RNA export pathway was described in a study that found a mutant in the E1 enzyme is defective in mRNA export in *S. pombe*. Subsequent studies using *S. cerevisiae* found that two HECT E3s, Tom1 and Rsp5, are the major E3 components in the ubiquitin-mediated RNA export pathway (48, 183). Additionally, a separate study using a genetic screen for isolating mutants with defective RNA export identified an *rsp5* mutant (166), further suggesting that Rsp5 promotes RNA export from the nucleus. Notably, Rsp5 seems to globally affect the export of nuclear RNAs, as the *rsp5* mutant shows a nuclear accumulation of tRNA and 60S pre-ribosomal subunits, as well as mRNAs. Although the biochemical mechanism remains unknown, it was shown that the nuclear processing of pre-tRNAs and pre-rRNAs are defective in the *rsp5* mutant (166), suggesting that Rsp5 may affect the processing step, which will be reflected as a defect in nuclear export. Interestingly, several proteins involved in ribosomal biogenesis and RNA processing were isolated as ubiquitinated proteins in a proteomic study (171), and some might be the direct substrates of Rsp5, as will be discussed in Chapter 4. A clue to the mechanism of Rsp5-mediated RNA export is provided in a recent study, where it was suggested that Hpr1, a member of the THO/TREX (transcription/export) complex that mediates mRNA transcription to nuclear export, is a target of the Rsp5-mediated ubiquitination and degradation (80). How the degradation of Hpr1 leads to the export of mRNAs remains unclear.

Mitochondrial inheritance

An *rsp5* mutant was identified in a genetic screen that showed failed distribution of Mod5, a tRNA-modifying enzyme, which normally is localized in the nucleus, cytoplasm, and mitochondria (254, 255). Meanwhile, an independent group isolated a suppressor of a *mdm1* mutant, which displays defect in mitochondrial distribution and morphology (64). The suppressor, named *smm1* (suppressor of mdp1-dependent mitochondrial inheritance defects 1), also map to *RSP5*. Interestingly, another suppressor, *smm2*, was found to be a mutation in *BUL1*, whose protein product binds to Rsp5 (244), suggesting that the Rsp5 and Bul1 complex are involved in the mitochondrial distribution pathway. Importantly, both *mdp1* and *smm1* mutations were mapped within the HECT domain of Rsp5, suggesting that the catalytic activity of the E3 ligase was somehow disrupted in those mutants, as is the case in the *rsp5-1* mutant (227). The *smm1* and *smm2* mutants displayed defects in mitochondrial distribution, as well as cells harboring mutations in both *ubc4* and *ubc5*, the two E2s known to work with Rsp5 in yeast (168), further suggesting that the Rsp5-mediated ubiquitinating activity is responsible for the mitochondrial distribution. Furthermore, the proper distribution of mitochondria has been shown to require K63-linked polyubiquitination, consistent with previous roles of Rsp5 in the endocytic pathways (66, 206). The specific substrates and molecular mechanism involved in the Rsp5-mediated regulation of mitochondrial distribution remain to be determined.

Other potential roles

As mentioned above, the genetic interaction between Rsp5 and Spt3 suggests that Rsp5 might have a role in transcriptional regulation. The most convincing data implicating Rsp5 in its potential role in the transcription might be that Rsp5 directly binds to and ubiquitinates Rpb1, the larges subunit of RNA polymerase II (107). Interestingly, a recent proteomic work with the aim of isolating TFIID complex has identified Rsp5 and Bul1 along with other transcriptional factors, suggesting that Rsp5 could be physically forming a complex with transcriptional machinery in the nucleus. However, the molecular details as to how Rsp5 might regulate RNA pol II-mediated transcription remain uncharacterized. Although it has been shown that Rsp5 induces degradation of Rpb1 in DNA-damaged cells (10), whether or not this occurs in undamaged cells is unknown.

Rsp5 has also been implicated in cell wall biogenesis (114). It has been shown that an *rsp5* mutant is hypersensitive to a cell destabilizing agent calcoflour white (CFW), suggesting that the cell wall is defective in the mutant. An electron microscopy study revealed an increased chitin level and an aberrant cell wall structure in the mutant, suggesting that Rsp5 might be involved in the regulation of cell wall biogenesis and/or chitin synthesis. Consistent with this, the temperature sensitive phenotype of the *rsp5-1* mutant was rescued by presence of the osmotic stabilizer sorbitol in the media (120, 244), further suggesting that the basis for the growth defect of the mutant in high temperature might be a defective cell wall. The implications of Ubp2 and K63-polyubiquitin linkages in this role of Rsp5 will be discussed in more detail in chapter 3.

Pathways	Substrates	Chain type/consequence
OLE pathway	Spt23, Mga2	Mono?/ proteolytic activation
Endocytosis	Gap1, Fur4, Ste2, Pma1, Put4 Smf1, Cps1, Phm5, Hxt6/7, Zrt1	K63, mono?/ endocytosis
RNA export	Hpr1?	K48?/ degradation
Mitochondrial inheritantce	Unknown	K63
Other pathways	Rpb1, Csr2, Ecm21, Rvs167 Vps9, Rpa1	K63, mono/ ? degradation (Rpb1)

Table 1.1 Known targets and polyubiquitin chain types of Rsp5.

1.7 Regulation of HECT E3s

Studies during the past decade showed that HECT E3s are involved in diverse physiological pathways from yeast to human. Although the specific substrates of the HECT E3s have been relatively well characterized, mechanisms for regulating the inherent catalytic ligase activities are only beginning to be explored. Regulation is achieved at different steps: phosphorylation, E2 interaction, or by associating with regulatory cofactors such as deubiquitinating enzymes. Here I summarize some of the recently uncovered mechanisms for regulating HECT E3 ligases.

Recruitment of E2

The TGF-β signaling pathway has pleiotropic effects on multiple cellular processes, including cellular differentiation and immune responses (135). Regulating the pathway is critical for achieving the balanced responses to cellular stimuli, and this can be achieved in multiple steps, including the down-regulation of the TGF-β receptor protein (135). It has been shown that Smurf2-mediated ubiquitination and degradation of TGF-β receptor proteins play a major role in inhibiting the signaling pathway (118). This study found that Smurf2, which is normally in the nucleus, is recruited to the membrane receptor by Smad7, one of the several Smad-family transducer proteins in the signaling pathway. Interestingly, a recent study from the same group have shown that the ubiquitinating activity of Smurf2 is significantly stimulated by Smad7 (169). This study found that the N-terminal domain of Smad7 binds to both the HECT domain and the E2, UbcH7, thereby facilitating interaction between the two proteins. The crystal structure of the HECT domain of Smurf2 revealed overall similarity in the

conformation the HECT domain to that of the two closely related HECT E3s, E6AP and WWP1. Based on this crystal structure, the superimposed view of the E2 binding surface between the Smurf2 HECT domain and that of E6AP was analyzed. Interestingly, it revealed that the key determinant hydrophobic residues (represented by Ile656 and Phe691 in E6AP) in the E2 binding groove within the Smurf2 HECT domain were replaced by non-conserved hydrophilic amino acids His547 and Tyr581, potentially explaining why Smurf2 has lower inherent E2 binding activity. Interestingly, when the key residues in the E2 binding pocket of the Smurf2 HECT domain were replaced by those of E6AP, the mutant Smurf2 became constitutively active, irrespective of Smad7, as assayed in auto-ubiquitination and the activity of TGF-ß receptor activity.

Therefore, Smad7 appears to have dual effects: it recruits Smurf2 to the substrate, the TGF-ß receptor, and it also stimulates the catalytic ubiquitinating activity of the ligase by recruiting E2. It is tempting to speculate that Smurf2 might have beeen designed to have an inherent sub-optimal level of E2 binding activity such that it can be stimulated only by associating with the positive regulator. However, this mechanism is not consistent with a recent finding, which demonstrated that the same determinant in the E2 is competed by E1 and E3, thus allowing the sequential E1-E2 and E2-E3 reactions (55). Thus, the increased affinity between the Smurf2 HECT and UbcH7 potentially means a decreased affinity between E1 and the E2, and a decreased ubiquitination. It will be interesting to know if Smad7 hinders the binding between UbcH7 and E1. It is not known whether other HECT E3s are regulated in a similar

manner. Interestingly, WWP1, a HECT E3 closely related to Smurf1 and Smurf2, have also been shown to bind to Smad7 and downregulate TGF-ß receptors (126). It is not known whether Smad7 also affects the E2 binding ability of the WWP1 HECT domain.

Modulating the ability of E2 recruitment as a means of regulating the E3 activity has been previously reported in a Cullin-based RING E3 ligase (119). It had been shown that neddylation of cullin subunits stimulate the ubiquitinating activities of the E3 complexes (132). The underlying mechanism for the activation of E3 by neddylation has been shown to involve increased recruitment of Ubc4, an E2, to the Cul1 subunit of the E3 complex, thereby increasing the ubiquitination of a substrate IkBα (119). How the E2 recognizes neddylated Cul1 is unknown.

Phosphorylation-mediated regulation

The HECT E3 ligase Itch is involved in numerous cellular processes including cell cycle control and immune process (143, 184, 185). The regulatory mechanism for Itch represents how activities of HECT E3 ligase can be modulated by different types of phsophorylation. It was shown that Itch can be either positively or negatively regulated, depending on different kinases and corresponding phosphorylation acceptor sites. It has been shown that Itch-mediated c-Jun degradation is dependent on JNK1 kinase, which phosphorylates Itch (68). The underlying molecular mechanism is reminiscent of that of the Src activation (152). It has been shown that Itch, which is normally kept inactive by an inhibitory intra-molecular interaction between the WW domains and the HECT domain, becomes activated when three residues (S199, T222, S232) within the so-called PRR (proline rich region) domain are phosphorylated by JNK (67). The

phosphorylation presumably triggers a conformational change that disrupts the inhibitory intra-molecular interaction, thus switching the E3 to an active mode. Interestingly, it turns out that phosphorylation does not always activate the ligase activity, but rather can be inhibitory. It has been shown that Itch can be negatively regulated by Fyn kinase-mediated phosphorylation on Y371 residue (242). The mechanism for this inhibitory effect seems to be different from the former case, as it was shown that the phosphorylation on Y371 reduces the E3's ability to bind to its substrates, although the exact mechanism remains unclear. Thus, the activity of Itch seems to be balanced by different signaling pathways that induce the phosphorylation at alternate residues.

Based on the sequence similarity among the HECT E3 members, other HECT E3s might be subject to a similar mechanism. Anther HECT E3 ligase, Nedd4, is involved in the ubiquitination and endosomal down-regulation of membrane receptor proteins, with the best characterized one being the Na (+) channel (ENaC). The Nedd4-mediated regulation of the receptor activity has been shown to be hormone-dependent, and it was shown that the down-regulation of ENaC was significantly inhibited by phosphorylation of two serine residues on Nedd4, which is mediated by hormone-induced kinase (41). The inhibitory effect of phosphorylation on the Nedd4 does not seem to inhibit the catalytic activity of the HECT domain, as was the case with Itch, but rather it was suggested to affect the ability of Nedd4 to bind to ENaC. Given that the phosphorylation sites locate between the WW domains, this study suggests that

phosphorylation might alter the conformation of the domains that interact with the PY motif of the substrate.

Deubiquitinating enzymes

Increasing evidence suggests that deubiquitinating enzymes (DUBs) play significant roles in many physiological pathways. It has been shown that some DUBs have their own substrate specificity to directly reverse the ubiquitination status of substrates (103, 136, 138). Interestingly, recent studies have found that some DUBs are physically associated with E3 ligases (17, 89, 120, 127, 220, 239). The purposes of the complex formation seem to be different in each case, as some DUBs reverse the autoubiquitination of E3 ligases for stabilization and some DUBs inhibit the E3 activity. Although many RING E3 ligases seem to be regulated by specific DUBs, the only HECT E3 that has been shown to associate with a DUB is Rsp5 (Chapter 2) (120). Rsp5, an essential HECT E3 in *S. cerevisae*, has been shown to associate with Ubp2, one of the 16 ubiquitin-specifc proteases (UBPs) in yeast. The functional relationship of the Rsp5 and Ubp2 complex will not be described in detail in this section, as it is the major subject of this dissertation. It is so far unknown whether Rsp5-homologs in mammalian cells (*e.g.*, Nedd4, Smurf1/2) also utilize a similar type of regulation, in association with DUBs.

Other mechanisms

Mule/ARF-BP1

ARF is a tumor suppressor that has been shown to stabilize p53 by inhibiting the Mdm2 E3 ligase (202). In another study that attempted to characterize the Mdm2-

independent tumor suppression mechanism of ARF, a HECT E3 ligase was identified to be as a major associating factor of ARF (23). Interestingly, the HECT E3, named Mule/ARF-BP1, has been shown to ubiquitinate and destabilize p53, (191). Notably, in this study, the ability of Mule/ARF-BP1 to ubiquitinate and destabilize p53 was strongly inhibited by ARF both *in vitro* and *in vivo*, suggesting that Mule/ARF-BP1 is a key target of ARF for p53 stabilization. So, how is the Mule/ARF-BP1 E3 activity regulated by ARF? Although the answer is not clear, ARF seems to inhibit the catalytic activity, not the substrate binding activity, as ARF was found in this study to inhibit even the auto-conjugating activity of the E3. It is intriguing to note that ARF was shown to directly bind to Mule/ARF-BP1 and the region required for the ARF binding in the E3 included the HECT domain. This raises the possibilities that ARF might inhibit E2-binding ability of the HECT domain, or it might limit the proper conformation of the HECT domain that is necessary for its catalytic activity. Other potential regulatory mechanisms may include Bul1 and Bul2-mediated polyubiquitination of Gap1, although the precise biochemical reactions remain unknown (discussed in "E4" section).

1.8 HECT E3s and clinical implications

HECT E3s are involved in many physiological processes in human cells and implicated in several human diseases, including Angelman syndrome, Liddle syndrome, cervical carcinoma, and others.

Angelman syndrome

Angelman Syndrome (AS) is a severe neurological disease with mental retardation, seizures, and motor dysfunction in human (34, 155). AS was originally found to be associated with chromosomal deletions of human chromosome 15q11-q13 (155). Although the mutations of other genes in the chromosomal region might contribute to the disease, the majority of the mutations were found to be within the *UBE3A* locus, which encodes E6AP (34, 42, 102, 155). Remarkably, only the maternal allele of E6AP is expressed in the brain due to tissue-specific maternal imprinting, and AS patients lack a functional maternal allele of E6AP in brain (224). Most of the AS-associated mutations in the E6AP gene were found to involve missense mutations, single amino acid insertions/deletions, or frameshift mutations that cause truncation of the protein. Importantly, most of these mutations map within, or cause entire truncation of the HECT domain, and the structural analysis of the HECT domain of E6AP revealed that many of the missense mutations map within the catalytic cleft (102, 155). This evidence strongly suggests that the abrogation of the catalytic activity of E6AP correlates with AS. However, the specific target(s) of E6AP in the brain associated with AS is yet to be identified.

Liddle syndrome

Liddle syndrome is a genetic disorder associated with abnormal sodium reabsorption in the distal tubule (230). The epithelial Na^+ channel (ENaC) plays a critical role in the salt and fluid homeostasis in cells and a deregulated opening of the channel has been known to cause Liddle syndrome (211, 230). It was shown that the

mutations that cause truncation of the cytoplasmic C-terminus or mutations in the conserved PY-rich sequences of the beta and gamma subunit of the epithelial sodium channel (ENaC) cause the disease. The PY sequences have been shown to interact with the WW domains of Nedd4 and the decreased interaction between Nedd4 and ENaC has been suggested to be the basis for Liddle syndrome (210, 211, 213, 230). The cells with the mutation in the PY motif of ENaC have been shown to display increased channel activities, suggesting that decreased ubiquitination of ENaC by Nedd4 is associated with the disease. In fact, it has been shown that the deletion of the PY motif of ENaC in Liddle syndrome correlates with decreased ubiquitination and increased retention of ENaC at the plasmamembrane (196, 210, 212). Consistent with these observations, ENaC stability has been shown to be increased by a lysosomal inhibitor, suggesting that Nedd4-mediated ubiquitination of ENaC leads to the endocytic degradation (211). There have yet been no reports that Liddle syndrome is associated with mutations in Nedd4. Although the reason for this is not clear, it is possible that more severe phenotypes may be associated with a defective catalytic activity of Nedd4.

Cervical cancer

The E6/E6AP-mediated degradation of p53 has been shown to be a critical event in the development of cervical carcinoma (106). The “high-risk” human papillomaviruses (HPV) such as HPV-16 and HPV-18, are associated with more than 95% of human cervical carcinomas, while the “low-risk” HPVs such as HPV-6 and HPV-11 are associated with benign tumors (256). In the HPV-positive cervical carcinomas, generally two HPV-encoded transforming oncoproteins are found, E6 and

E7. The oncoproteins are necessary and sufficient for immortalizing human primary keratinocytes and inactivate two critical cellular tumor-suppressive proteins, p53 and pRB, respectively (100). A study has shown that the mechanism for the E6-mediated inactivation of p53 involves the ubiquitin/proteasome pathway (194). It was shown that E6 binds to a cellular HECT E3, E6AP, and hijacks the E3 ligase activity of E6AP for ubiquitinating p53, which is not a normal target of the E3 ligase (106, 217). siRNA-mediated depletion of either E6 or E6AP significantly stabilized p53 in the HPV-positive HeLa cell lines, further suggesting that this is the primary mechanism for inactivation of p53 in cervical cancer (122). Although E6 has been shown to target Myc for degradation independent of E6AP (77), E6AP has been shown to be required for majority of the E6-mediated degradation of cellular proteins other than p53 (69, 71, 130, 165). Consistent with this, a comprehensive microarray analysis has shown that siRNA-mediated depletion of E6 or E6AP resulted in nearly identical alterations in total transcriptional profiles in three different HPV-positve cell lines, suggesting that E6AP mediates a broad range of E6-mediated functions (122).

Others

Nedd4-mediated ubiquitination of VP40, a structural protein of Ebola virus, has been shown to be an essential process for the viral budding and similar mechanisms have been suggested to be used for many other retroviruses (81, 123, 245). TGF-ß signaling, which is critical for the broad spectrum of physiological processes including cell proliferation and differentiation, has shown to be downregulated by Smurf1, Smurf2, and Itch (13, 131, 249). Itch has also been shown to directly ubiquitinate and

down-regulate p63 and p73, two p53-related proteins that play important roles in cellular development and cell cycle control (184, 185). Additionally, Itch knockout-mice have been shown to display serious immunological defects, such as hyperplasia of lymphoid cells and pulmonary chronic interstitial inflammation (172), suggesting its critical role in the immune system. Mule/ARFBP-1 poses as a potential therapeutic target for rescuing p53 from degradation to induce apoptosis of tumor cells (22, 23). Finally, Herc5, an interferon- induced HECT E3, has been shown to play a crucial role in the conjugation of ISG15 (interferon-stimulated gene 15) (39, 236), a ubiquitin-like molecule that appears to play roles in anti-viral mechanisms (170, 248). Therefore, understanding the regulatory mechanisms of HECT E3s will be important for a broad range of clinical purposes.

CHAPTER TWO

Biochemical and Genetic Analysis of the Rsp5/Rup1/Ubp2 complex

2.1 Introduction

RSP5 is one of five *Saccharomyces cerevisiae* genes encoding HECT E3s and is the only one that is essential under normal growth conditions (227). Lethality of the *rsp5*Δ mutations can be suppressed by the addition of oleic acid to the growth media (99). The basis of oleic acid rescue is Rsp5- mediated ubiquitination of the Spt23 transcription factor, which activates Spt23 by stimulating a proteasome-catalyzed processing event (181). Processed Spt23 activates transcription of the *OLE1* gene, encoding a fatty acid desaturase. Rsp5 has been implicated in other cellular processes, most notably in trafficking of plasma membrane proteins. Rsp5 ubiquitinates several integral plasma membrane proteins, including Fur4 (66), Gap1 (84), and Ste2 (50), targeting them for ubiquitin-mediated endocytosis, and can also direct ubiquitin-mediated trafficking of proteins from the trans-Golgi network (TGN) to the vacuole (86). The latter requires the function of Bul1 and Bul2, two closely related proteins that interact with Rsp5 and influence some functions of Rsp5 (4, 113).

A large family of deubiquitinating enzymes (DUBs) has the capacity to influence the fate of ubiquitinated proteins (4). There are at least three broad functions for deubiquitinating enzymes: 1) the processing of ubiquitin precursor proteins to generate mature ubiquitin, 2) reversing the polyubiquitination of substrate proteins (25, 139), and 3) facilitating ubiquitin removal at the proteasome, allowing target proteins to be translocated into the proteasome (4). DUBs are therefore capable of either promoting or antagonizing ubiquitin- and proteasome-dependent processes.

While several direct targets and functions of Rsp5 have been identified, the basis of regulation of Rsp5 activities is largely unknown. In order to gain insight into possible control or regulation of Rsp5 activity, we affinity purified Rsp5 along with associated proteins using the tandem affinity purification (TAP) method under conditions where interacting or regulatory proteins can be co-purified (177). A prominent Rsp5-associated protein was the Ubp2, a member of the UBP family enzyme. Rup1, a UBA (ubiquitin-associated) domain-containing protein, was found to mediate the Rsp5–Ubp2 interaction, and we present both biochemical and genetic evidence that Ubp2 and Rup1 complex antagonizes catalytic activity of Rsp5. Furthermore, I propose that the UBA domain of Rup1 has a role in facilitating the catalytic activity of Ubp2. Together, our in vitro and in vivo results indicate that at least a fraction of Rsp5 exists in a complex with Ubp2, and that the Ubp2/Rup1 complex serves to antagonize, and potentially regulate, Rsp5 *in vivo*.

2.2 Materials and methods

Yeast strains, media, and plasmids

A list of yeast strains is shown in Table 2.1. Growth media containing oleic acid contained 0.5mM oleic acid (Sigma) in the presence of 1% Tergitol. The *NTAP-RSP5* strain (YK001) was generated using FY56 as the parental strain. A C-terminally truncated RSP5 mutant fragment was subcloned into pYES2 vector (Invitrogen) deleted of the 2u origin, with 500 bp of the RSP5 upstream sequence inserted in place of *PGAL1*. The construct was transformed into FY56 and colonies were selected on Ura- plates. Clones that expressed full-length NTAP-Rsp5 and truncated (untagged) Rsp5 protein were screened by anti-Rsp5 immunoblotting. Clones were then selected on uracil/5-FOA-containing plates, and clones that expressed NTAP-Rsp5 as the sole source of Rsp5 were isolated (strain YK001). Deletion of the complete *UBP2* or *RUP1* ORFs (stains YK003, YK004, and YK006) was carried out using a PCR-based method with KanMX6 selection (144). C-terminal 3xHA tagging of genomic UBP2 in YK001 and BY4741 strains (YK002 and YK008, respectively) was carried out similarly, using a HIS3 marker. Fulllength ORFs of *HA-UBP2*, *HA-RUP1*, *HA-UBP3*, *HA-UBP4*, and *Flag-SPT23* were PCR-amplified from yeast genomic DNA. The active-site mutation of UBP2 (ubp2-C745S) was generated by site-directed mutagenesis. Other *rsp5, ubp2*, and *rup1* mutants were generated by PCR amplification from each full-length ORF. All ORFs were subcloned into pYES2 vector for *in vitro/in vivo* expression or pGEX-6p-1 vector (Amersham Biosciences) for bacterial expression of GST fusion proteins.

TAP protein purification

The TAP purification procedure was similar to that described previously (177). An 8 L culture of strain YK001 was grown in YPD to an OD600 of 1.2, at 30C. The cell pellet was resuspended in NP-40 lysis buffer (0.5% NP-40, 150mM NaCl, 10mM Tris 8.0, supplemented with protease inhibitors) and cells were disrupted with a bead beater. The extract was cleared by centrifugation at 31000 g for 15 min at 4C. IgG Sepharose (Amersham Biosciences) was added and incubated with mixing for 4 h at 4C. The beads were washed in NP-40 lysis buffer, followed by TEV cleavage buffer (0.1% NP-40, 150mM NaCl, 10mM Tris pH 8.0, 0.5mM EDTA, 1.0mM DTT), and TEV protease was added and incubated at room temperature for 2 h. The supernatant was diluted with calmodulin binding buffer (150mM NaCl, 10mM Tris pH 8.0, 1mM magnesium acetate, 1mM imidazole, 2mM CaCl2, 10mM β-mercaptoethanol) and Calmodulin Sepharose (Amersham Biosciences) was added and incubated for 1.5 h at 41C. After washing, bound proteins were eluted in buffer containing 150mM NaCl, 10mM Tris pH 8.0, 0.02% NP-40, 1mM Mg^{2+} acetate, 1mM imidazole, 20mM EGTA, 10mM DTT). The final eluate was TCA-precipitated, resuspended in 1X SDS–PAGE loading buffer, and loaded on a 4–15% gradient SDS–PAGE gel. Gels were stained with either silver or Coomassie blue. Bands were excised from a Coomassie blue-stained gel and subjected to in-gel tryptic digest. The fragmented peptides were analyzed by LC/MS. The peptide sequence information was used to search for protein identification using the Mascot (Matrix Sciences) search engine. For confirmation of protein interactions, small-scale TAP purifications were performed. Cell extracts from each TAP strain was subject to IgG

Sepharose purification, with release of bound proteins by SDS–PAGE loading buffer. Proteins were separated by SDS–PAGE and immunoblotting was performed using the indicated antibodies.

In vitro protein interaction assays

GST fusion proteins were expressed from the pGEX-6p-1 vector in *Escherichia coli* DH5a and purified by standard methods on glutathione Sepharose. 35S-labeled proteins were synthesized *in vitro* using a coupled transcription–translation rabbit reticulocyte system (Promega). Yeast cell extracts for binding assays were prepared by growing cells in YPD until mid-log phase, resuspending the pellets in NP-40 lysis buffer, and lysing the cells with a bead beater. Cell extracts were cleared by centrifugation at 27,000 g for 10 min. Either 50 mg of whole-cell extracts was used for each binding assay or equal volumes of fractions from a DEAE ion exchange column. Complex formation using purified proteins (Rsp5, Ubp2, and Rup1 or Rup1ΔUBA) was performed using GST fusions. Ubp2, Rup1, and Rup1ΔUBA were cleaved from GST by PreScission protease (Amersham Biosciences). Binding reactions were performed using GST-Rsp5 on glutathione beads and each of the other free proteins for 2 h at 4C. Total protein was recovered from the washed beads by elution with SDS–PAGE loading buffer. For mapping of domains that mediate the ternary complex, bacterially expressed and purified GST-Rsp5 proteins (227) and *in vitro* translated wild-type Ubp2 were used, along with purified Rup1 protein from bacteria. To map regions of Rsp5 that are required for binding to Rup1, bacterially purified GST-Rup1 and *in vitro* translated Rsp5 proteins were used.

To map the regions of Ubp2 that are required for binding to Rsp5 or Rup1, purified GST-Rsp5, Rup1, and *in vitro*-translated Ubp2 proteins were used. Binding reactions were performed as described above.

In vitro ubiquitination and deubiquitination assays

In vitro ubiquitination/deubiquitination assays were performed in the presence of 10mM Tris pH 7.5, 50mM NaCl, 5mM ATP, 5mM MgCl2, 0.1mM DTT, and 50 ug/ml ubiquitin (Sigma). Bacterially expressed wild-type Rsp5, Rsp5-C777A, Ubp2, Rup1, Rup1ΔUBA, and yeast E2 (Ubc1) were purified on glutathione Sepharose and cut with PreScission protease (Amersham Biosciences). WBP2, p53, hScribble, and Spt23 were *in vitro* translated in the presence of ^{35}S-methionine. The translation reaction (3–4 ul) was used for each ubiquitination/deubiquitination reaction. The ubiquitination reactions were carried out for 30 min at room temperature, followed by additional 30 min incubation in the presence or absence of Rup1 and/or Ubp2. The reactions were performed as described above and stopped by addition of 1X SDS–PAGE loading buffer and products were analyzed by SDS–PAGE and autoradiography.

Gel Filtration analysis

30ml of *TAP-UBP2* (YK005) strain was grown in YPD media until O.D of 1.5 and the cell extracts were prepared in a lysis buffer (0.1% NP40, 20mM NaCl, 10mM Tris pH. 7.0). The cell extracts were subject to 30ul of DEAE sepahrose (Amersham Biosciences) and bound for 20 minutes and washed with the same buffer 3 times before eluted with

500mM NaCl. The high salt eluate was subject to Sephacryl^{TM} High resolution S300 size exclusion column (Amersham Biosciences) and equal amounts were collected using high salt elution buffer (1M NaCl, 20mM Tris pH 7.0). Equal amount of each fraction was analyzed by 10% SDS-PAGE followed by western blot analysis using anti-Rsp5 or anti-TAP (peroxidase anti-peroxidase, Rockland) antibody.

Strain	Genotype	References
FY56	MATα *his4-912δR5 lys2-128δ ura3-52*	(107)
YK001	MATα *NTAP-RSP5 his4-912δR5 lys2-128δ ura3-52*	(120)
YK002	MATα *NTAP-RSP5 ubp2Δ:: UBP2-3XHA:: KanMX6 his4-912δR5 lys2-128δ ura3-52*	(120)
YK003	MATα *rsp5-1 ubp2Δ::KanMX6 his4912δR5 lys2-128δ ura3-52*	(120)
YK004	MATα *rsp5-1 rup1Δ::KanMX6 his4912δR5 lys2-128δ ura3-52*	(120)
FW1808	MATα *rsp5-1 his4912δR5 lys2-128δ ura3-52*	(107)
YK003	MATα *rsp5-1ubp2::KANMX6 his4912δR5 lys2-128δ ura3-52*	(120)
YK004	MATα *rsp5-1rup1::KANMX6 his4912δR5 lys2-128δ ura3-52*	(120)
BY4741	MATa *his3 leu2 met15 ura3*	Open Biosystems
YK005	MATa *TAP-UBP2 his3 leu2 met15 ura3*	Open Biosystems
YK006	MATa *TAP-UBP2::HIS3 rup1::KanMX6 his3 leu2 met15 ura3*	(120)
YK007	MATa *TAP-RUP1::HIS3 his3 leu2 met15 ura3*	Open Biosystems
YK008	MATa *ubp2*Δ*::UBP2::3XHA::KanMX6 his3 leu2 met15 ura3*	(120)
YK009	MATa *ubp2*Δ*::KanMX6 his3 leu2 met15 ura3*	Open Biosystems
YK010	MATa *rup1*Δ*::KanMX6 his3 leu2 met15 ura3*	Open Biosystems
YK011	MATa *TAP-UBP3 his3 leu2 met15 ura3*	Open Biosystems
YK012	MATa *TAP-UBP4 his3 leu2 met15 ura3*	Open Biosystems

Table 2.1 List of the yeast strains used in chapter 2.

2.3 Results

Purification of NTAP-Rsp5 and associated proteins

To identify proteins associated with Rsp5, I utilized a TAP-purification method. The advantage of the TAP method over other conventional methods such as yeast two hybrid screen is that both indirectly and directly interacting proteins can be purified under physiologically relevant conditions. Additionally, the TAP method can indicate the approximate stoichiometry of proteins in a given complex. A yeast strain was generated in which the NTAP (N-terminal TAP) epitope was integrated at the 5' end of the chromosomal *RSP5* ORF, so that expression of NTAP-Rsp5 was directed by the natural RSP5 promoter (Figure 2.1A; see material and methods). The *NTAP-RSP5* strain was fully viable at both 30C and 37C, and the expression level of NTAP-Rsp5 was similar to that of endogenous Rsp5 in the parental strain (Figure 2.1B). NTAP-Rsp5 protein was purified from extract of an 8 L culture grown to mid-log phase. The first affinity purification step was performed on IgG Sepharose, with release of NTAP-Rsp5 by cleavage with TEV protease. The second affinity step was performed on calmodulin agarose with EGTA elution. The final eluate was concentrated and an aliquot was analyzed by SDS–PAGE and silver staining (Figure 2.1C). Several prominent bands in addition to Rsp5 were evident, and several were gel-isolated and identified by liquid chromatographic mass spectrometric analysis (LC/ MS). The major bands less than 45 kDa were all breakdown products of NTAP-Rsp5, and the band at approximately 95 kDa was full-length Rsp5 (containing the residual CBP epitope).

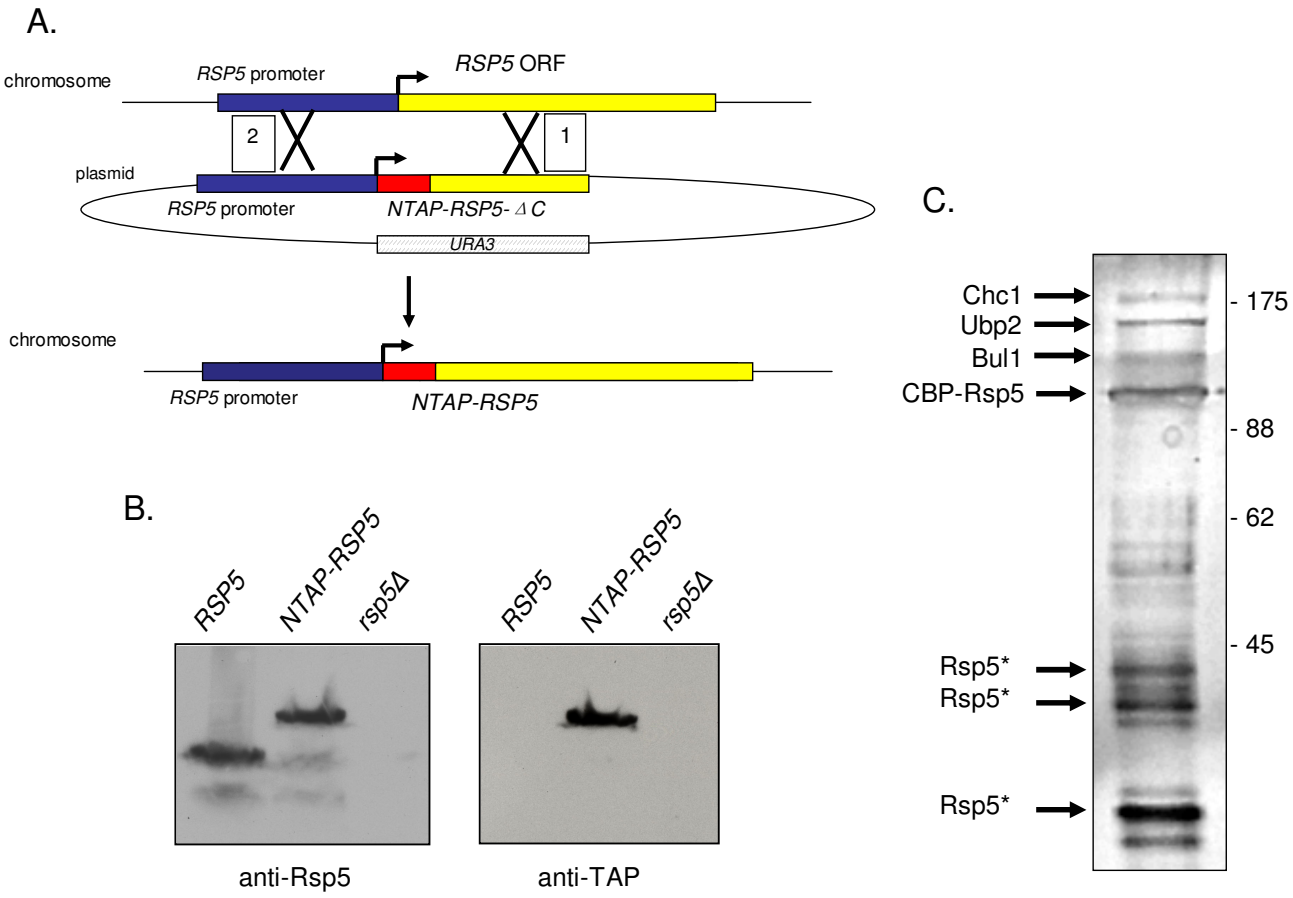


Figure 2.1 TAP-Rsp5 purification. (A) Shematic diagram for generating *NTAP-RSP5* strain. The scheme exploited two homologus recombination events, with first positive selection on Ura- plates (1) followed by second negative selection on 5FOA plates. (B) Confirmation for expression of NTA-Rsp5 using anti-Rsp5 and anti-TAP (peroxidase anti peroxidase). (C) Purification of NTAP-Rsp5 and associated proteins from extract of an 8 l culture (YK001). The final eluate was concentrated and separated by SDS–PAGE and silver stained. The positions and identities of bands identified by LC/MS are indicated; Rsp5* indicates breakdown products of NTAP-Rsp5. Molecular weight markers (kDa) are indicated.

The three prominent bands that migrated with apparent molecular weights greater than Rsp5 were identified as Bul1, Ubp2, and Chc1. Bul1 is a previously identified Rsp5-interacting protein (243, 244). Chc1 is clathrin heavy chain and it has been previously suggested that Chc1 might interact with Rsp5 in a Pan1-dependent manner (231). We were intrigued by the fact that Ubp2 co-purified with Rsp5, since these enzymes have opposing biochemical activities. Ubp2 is one of the 16 ubiquitin specific proteases (UBPs) and sequence analysis revealed that it has a three short stretches of sequences that are conserved among UBPs and UCHs (ubiquitin C-terminal hydrolases; shown as 'UCH' in the figure) at the C-terminal region of the protein, and large N-terminal extension with no distinctive sequence homology (Figure 2.2). One of the three conserved regions contains a putative Cys box domain with highly conserved sequences surrounding a cystein residue, suggesting that the cystein residue (C745 in Ubp2) is the catalytic active site.

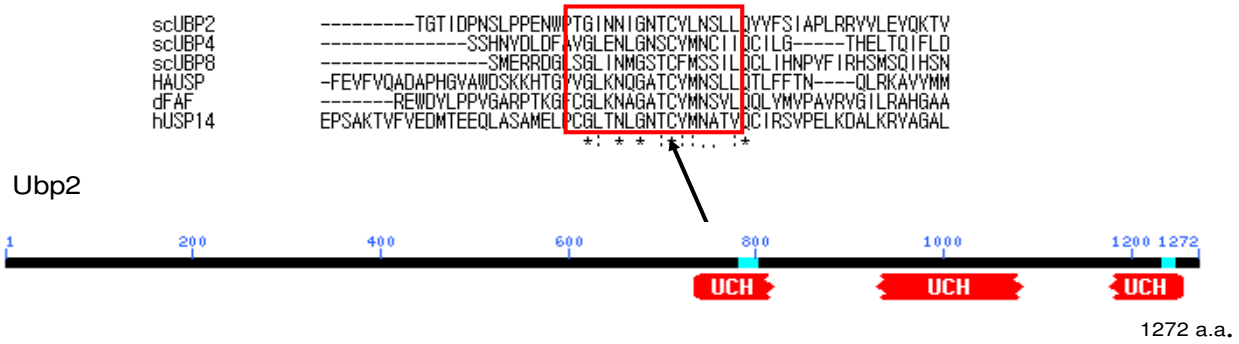


Figure 2.2 Ubp2 has a Cys box, one of the conserved signatures of UBPs.

The interaction between Rsp5 and Ubp2 was confirmed by integrating an HA epitope at the 3' end of the chromosomal Ubp2 ORF in both the *NTAP-RSP5* and parental *RSP5* strains. Extracts from these strains were subject to IgG Sepharose affinity chromatography, and bound proteins were analyzed by SDS–PAGE and immunoblotting with anti-HA antibody. Figure 2.3A shows that HA-Ubp2 specifically co-purified with NTAP-Rsp5, not with IgG sepharose in the control lane (lane 6). In addition, a strain expressing CTAP-Ubp2 (C-terminal TAP epitope) was used to demonstrate that endogenous Rsp5 protein co-purified with CTAP-Ubp2 (Figure 2.3B), not with IgG sepharose. These results strongly suggest that at least a fraction of the cellular pools of Rsp5 and Ubp2 exist in a stable complex *in vivo*.

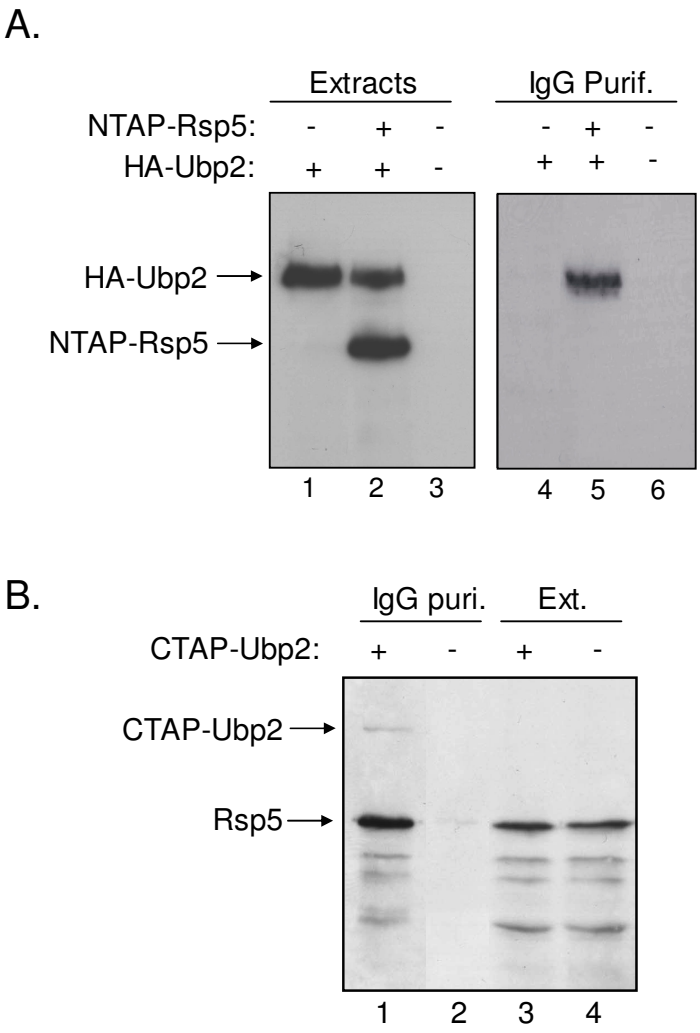


Figure 2.3 Confirmation of the Rsp5-Ubp2 interaction. (A) Extracts were prepared from YK002 (*NTAP-RSP5*, *HA-UBP2*), YK008 (*RSP5*, *HA-UBP2*), and BY4741 (*RSP5, UBP2*). Proteins were affinity selected on IgG Sepharose and bound proteins were released with SDS–PAGE loading buffer. Total extracts (lanes 1–3) and eluates (lanes 4–6) were analyzed by SDS–PAGE and immunoblotted with anti-HA antibody. (B)

Extracts were prepared from YK005 (*TAP-UBP2*) and BY4741 (*RSP5, UBP2*) and proteins were affinity selected on IgG Sepharose. Total extracts (lanes 1 and 2) and eluates (lanes 3 and 4) were analyzed by SDS–PAGE and immunoblotting with anti-Rsp5 antibody.

The Rsp5–Ubp2 interaction is mediated by Rup1

To determine whether Rsp5 and Ubp2 bound to each other directly *in vitro*, I performed binding assays with bacterially expressed GST-Rsp5 and *in vitro*-translated ^{35}S-labeld Ubp2. GST-Rsp5 is enzymatically active and has been shown to bind to several Rsp5-interacting proteins *in vitro* (107, 189, 199), yet stable interaction was not detected with the labeld Ubp2 (Figure 2.4A, lane 1). The addition of total yeast cell extract or a high-salt DEAE fraction from cell extract stimulated the binding of Ubp2 to Rsp5 (Figure 2.4A, lanes 2 and 5), suggesting that an additional factor(s) might mediate the interaction. GST-E6AP, a human HECT E3, did not bind to Ubp2 in the absence or presence of yeast cell extract. To identify the potential mediator of Rsp5–Ubp2 binding, I performed a large-scale purification of CTAP-tagged Ubp2 (C-terminally TAP tagged) and identified copurifying proteins. As expected, Rsp5 was identified in the LC/MS analysis of CTAP-Ubp2-associated proteins (Figure 2.4B). A prominent Ubp2-associated protein with an apparent molecular weight of approximately 85 kDa was identified by mass spectrometry as Rup1 (YOR138C; calculated molecular weight 75 kDa). Rup1 has an N-terminal UBA domain (amino acids 1–41) but no other discernible functional domains. UBA domains of some proteins have been shown to

bind to polyubiquitin chains (24, 179, 233). *RUP1* has been reported to be a nonessential gene Saccharomyces Genome Database, www.yeastgenome.org), a result that we confirmed (below). In a separate experiment, I performed a large-scale CTAP-Rup1 purification and Ubp2 was purified as nearly one to one stoichiometric amount in the purification by LC/MS analysis (not shown). Rsp5 was also found in the analysis.

To confirm the Ubp2–Rup1 interaction, HA-Rup1 was expressed in the CTAP-Ubp2 strain and was shown to copurify with CTAP-Ubp2 (Figure 2.4C). HA-Rup1 also co-purified with Rsp5 when expressed in the *NTAP-RSP5* strain (Figure 2.4C). Rup1 was not identified in the NTAP-Rsp5 purification (Figure 2.1C), due to the fact that the region of the gel where Rup1 migrated was not analyzed in detail because of the large amount of breakdown products of NTAP-Rsp5 protein that migrated in the 80–90 kDa range. To determine whether Rup1 was responsible for mediating the Rsp5–Ubp2 interaction, a *rup1*Δ mutation was created in the CTAP-UBP2 strain. CTAP-Ubp2 was purified from this strain, and immunoblotting indicated that the degree of Ubp2–Rps5 association was greatly decreased relative to the equivalent *CTAP-UBP2/RUP1* strain (Figure 2.5A, compare lanes 1 and 4). Figure 2.5A also shows that Rsp5 did not significantly co-purify with two other TAP-tagged Ubp proteins, Ubp3 or Ubp4, strongly suggesting that the interaction of Rsp5 with Ubp enzymes is specific for Ubp2. In addition, *RUP1* WT yeast cell extract stimulated the binding of *in vitro* translated Ubp2 to GST-Rsp5, while cell extract from an *rup1*Δ strain did not (Figure 5B).

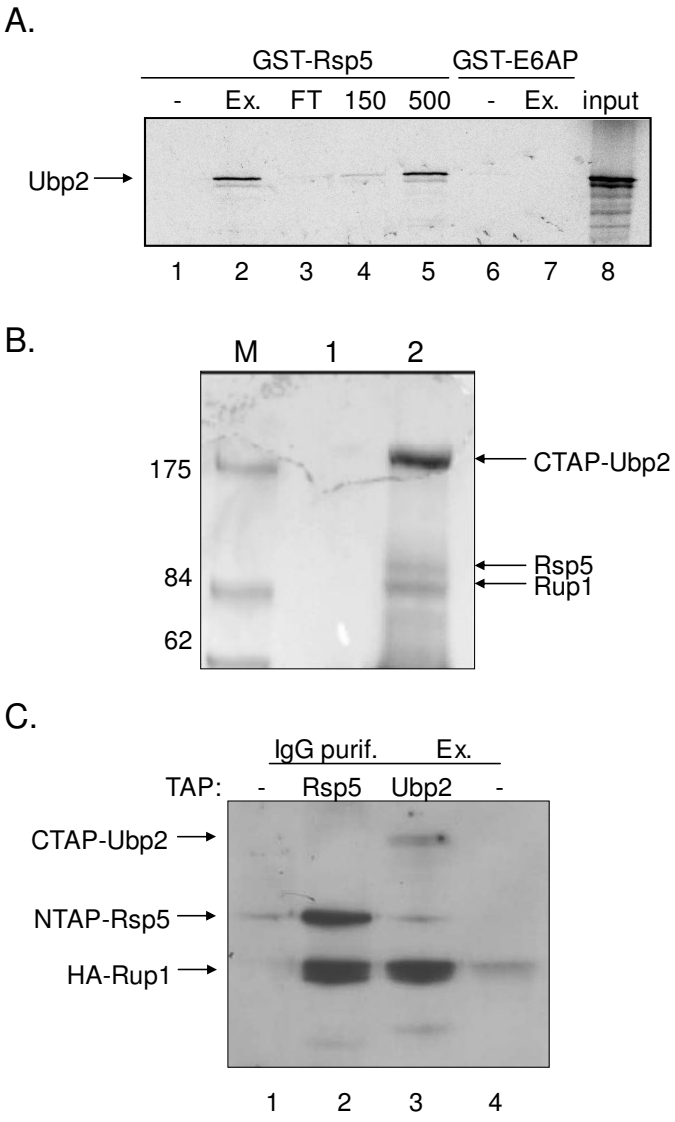


Figure 2.4 A cellular factor mediates the association of Rsp5 and Ubp2. (A) *In vitro*-translated ^{35}S-labelled Ubp2 was incubated with GST-Rsp5 or GST-E6AP immobilized on glutathione Sepharose, in the absence (–lanes) or presence of cell extracts (Ex.; lanes 2 and 7) or fractionated extracts (lanes 3–5) from the *ubp2*Δ strain. Bound Ubp2 was

detected by SDS–PAGE and autoradiography. DEAE high-salt fractions were either 150 or 500 mM NaCl eluates; FT represents the flow-through fraction. Input (lane 8) shows 50% of translation mixture used in the binding assays. (B) TAP purification of Ubp2-associating proteins. Proteins from YK005 (*CTAP-UBP2*) were affinity selected on IgG Sepharose followed by elution and cleavage with TEV protease. The eluate (lane 2) was separated on 4–15% gradient gel and stained with Coomassie blue. A parallel purification was performed using the control BY4741 strain (lane 1). Arrows indicate the position of Ubp2, Rsp5, and Rup1 proteins, as identified by LC/MS. Molecular weight markers (kDa) are indicated. (C) HA-Rup1 binds to both NTAP-Rsp5 and CTAP-Ubp2. HA-Rup1 was expressed from a galactose-inducible promoter plasmid in strains YK001 (*NTAP-RSP5*), YK005 (*CTAP-UBP2*), and BY4741 (control, lane 1, not expressing any TAP-tagged protein). Extracts were prepared and TAP proteins purified on IgG Sepharose. Eluates (lanes 1–3) were analyzed by SDS–PAGE and immunoblotting with anti-HA antibody. Lane 4 shows HA-Rup1 in total lysate from BY4741. Detection of NTAP-Rsp5 and CTAP-Ubp2 (lanes 2 and 3, respectively) was a result of anti-mouse IgG secondary antibody recognizing the TAP epitope.

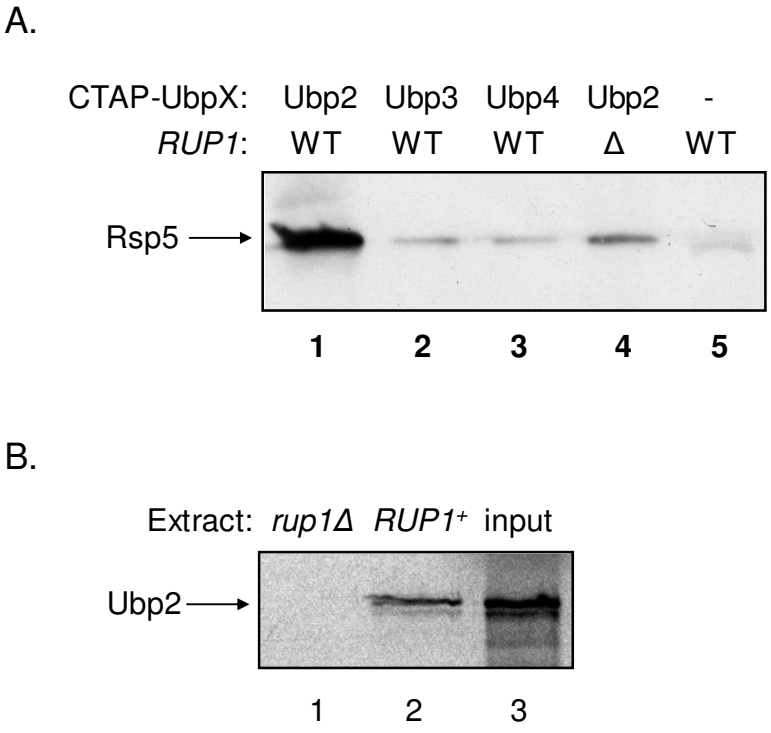


Figure 2.5 Rup1 is necessary for the association of Rsp5 and Ubp2 both *in vitro* and *in vivo*. (A) Cell extracts were prepared from strains expressing the indicated CTAP-tagged Ubp proteins in either an *RUP1* or *rup1*Δ background. Proteins were affinity selected on IgG Sepharose and eluates were analyzed by SDS–PAGE and immunblotting with anti-Rsp5 antibody. (B) ^{35}S-labeled Ubp2 was assayed for binding to GST-Rsp5 (as in Figure 4A) in the presence of cell extract from either the *rup1*Δ strain (lane 1) or an *RUP1* wild-type strain (lane 2). Lane 3 shows 50% of the input amount of Ubp2 used in the binding reactions.

To determine if Rup1 was sufficient for mediating the association in a purified system, Rsp5, Ubp2, and Rup1 were individually expressed as GST fusion proteins in bacteria. The proteins were purified and the GST moieties were removed from Ubp2 and Rup1 by site-specific proteolysis. GST-Rsp5, on glutathione sepharose, did not stably bind to purified Ubp2 (Figure 2.6A, lane 1). The addition of purified Rup1 stimulated Ubp2 binding (lane 4), indicating that Rup1 is sufficient for mediating the Rsp5-Ubp2 interaction. Furthermore, GST-Rsp5 bound directly to Rup1 in the absence of Ubp2, indicating that the interaction between these two proteins is direct. These experiments were also performed with a truncated form of Rup1, lacking the N-terminal UBA domain (Rup1-ΔUBA; lacking amino acids 2–40). Rup1-ΔUBA protein bound to Rsp5 similarly to full-length Rup1 and also mediated the interaction with Ubp2. Figure 2.6B confirms that GST-Rup1 binds to *in vitro*-translated Rsp5 in the absence of Ubp2, as well as to *in vitro* translated Ubp2 in the absence of Rsp5. GST-E6AP did not interact with either Rup1, Ubp2, or Rsp5. Together, the *in vitro* binding results indicate that Rup1 can interact directly with both Rsp5 and Ubp2, as well as simultaneously with both proteins.

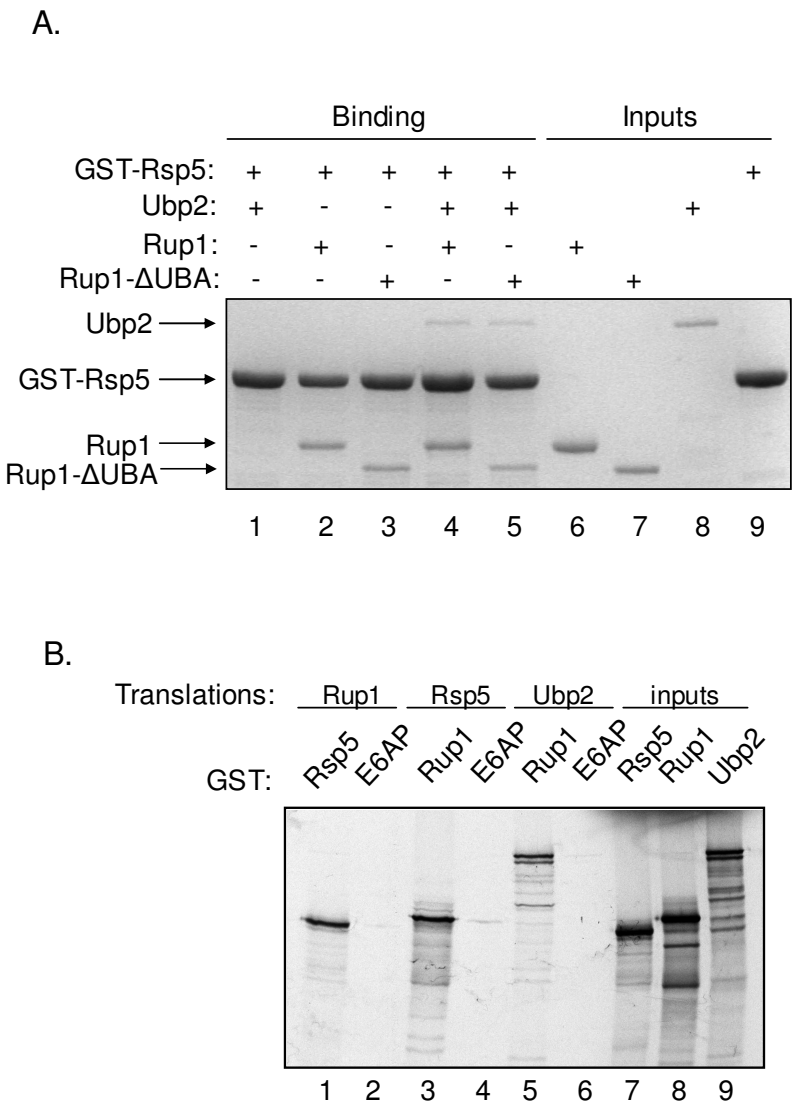


Figure 2.6 Rup1 mediates the interaction between Rsp5 and Ubp2. (A) Purified Ubp2, Rup1, and Rup1ΔUBA proteins were used in GST-Rsp5 pull-down assays. A 100% of input amounts of each protein is shown in lanes 6–9. Bound proteins were analyzed by SDS–PAGE and Coomassie blue staining. (B) Rup1, Rsp5, and Ubp2 were ^{35}S-labeled by *in vitro* translation and assayed for binding to purified GST-Rsp5, E6AP, or Rup1. Inputs (lanes 7–9) show 50% of the translation mixture used in the binding assays.

Domains that mediate formation of the ternary complex

To define the domains of Rsp5 necessary for binding to Rup1 and Ubp2, a series of N- and C-terminally truncated Rsp5 proteins were expressed either as GST fusion proteins or as *in vitro* translated proteins (Rsp5 proteins A–I; Figure 2.7A). The smallest region of Rsp5 that is capable of interacting with GST-Rup1 required both the C2 domain and the first WW domain (Rsp5-H; Figure 2.7B). Figure 2.7C shows binding of *in vitro* translated Ubp2 to GST-Rsp5 proteins A–E in the presence of purified Rup1 protein. Full-length GST-Rsp5 bound to Ubp2, but deletion of either the HECT domain or the region spanning the C2 domain abrogated Ubp2 binding. This is the only case where the HECT domain has been shown to be required for a protein-protein interaction, except for E2, although the HECT domain itself was not sufficient for interacting with Ubp2. Therefore, determinants for Ubp2 interaction span a large region of Rsp5 and extend beyond what is sufficient for binding to Rup1, suggesting that there might be a direct contact between Rsp5 and Ubp2, potentially in the HECT domain. In addition, Rsp5 deleted of the last six amino acids (Rsp5-E), which disrupts ubiquitination activity (189), as well as full-length Rsp5 containing the active-site Cys (C777) to Ala mutation (not shown), bound to Ubp2 similarly to wild-type Rsp5, indicating that catalytic activity of Rsp5 is not necessary for Ubp2 association. Similarly, Ubp2 with active-site Cys (C745) mutated to Ser equally bound to GST-Rsp5 compared to wild type Ubp2 (not shown), suggesting that the catalytic activity of Ubp2 is not required for the binding, either.

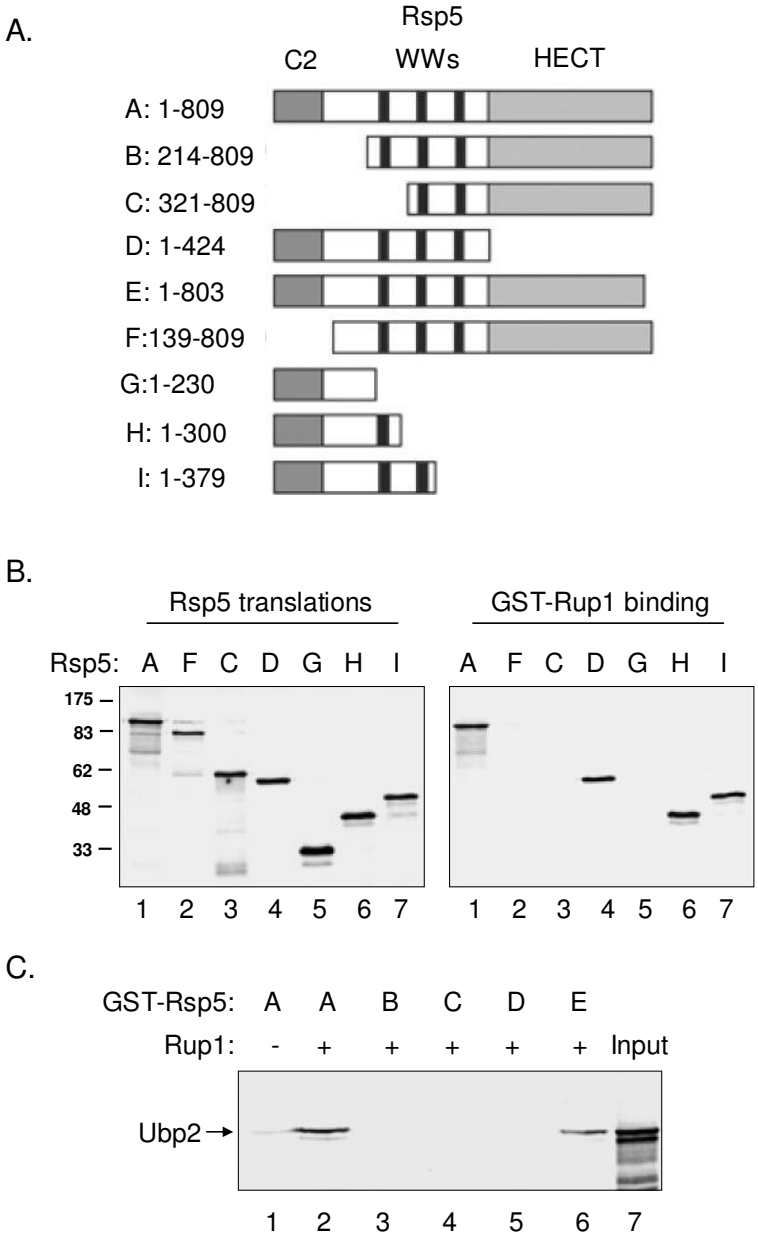


Figure 2.7 Domains of Rsp5 required for binding to Rup1 and Ubp2. (A) Schematic of Rsp5 truncation mutants used in binding assays. (B) Binding of GST-Rup1 to Rsp5 proteins. ^{35}S-labeled *in vitro*-translated Rsp5 proteins were incubated with GST-Rup1

and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel). (C) Binding of GST-Rsp5 proteins to Ubp2. Purified GST fusion Rsp5 proteins, on glutathione Sepharose, were incubated with ^{35}S-labeled *in vitro*-translated Ubp2 in the presence (lanes 2–6) or absence (lane 1) of purified Rup1, and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input (lane 7) indicates 50% of translation used in the binding reaction.

A series of N-terminally and C-terminally truncated Ubp2 proteins (Ubp2 proteins A–E; Figure 2.8A) were expressed by *in vitro* translation and assayed for GST-Rup1 binding (Figure 2.8B) and GST-Rsp5 binding in the presence of Rup1 (Figure 2.8C). The C-terminal 322 residues of Ubp2 (Ubp2-E) were sufficient to mediate direct binding to Rup1, and the same region was sufficient for Rup1-mediated Rsp5 association. Together with the results shown in Figure 2.6A, I conclude that (1) the UBA domain of Rup1 is not necessary for formation of the ternary complex, (2) the C-terminal 322 residues of Ubp2 are sufficient for interaction with both Rup1 and the Rsp5/Rup1 complex, (3) the region spanning the C2 domain and first WW domain of Rsp5 are sufficient for interaction with Rup1, and (4) in addition to determinants required for Rup1 binding, determinants within the HECT domain are also necessary for stable association of Rsp5 with Ubp2.

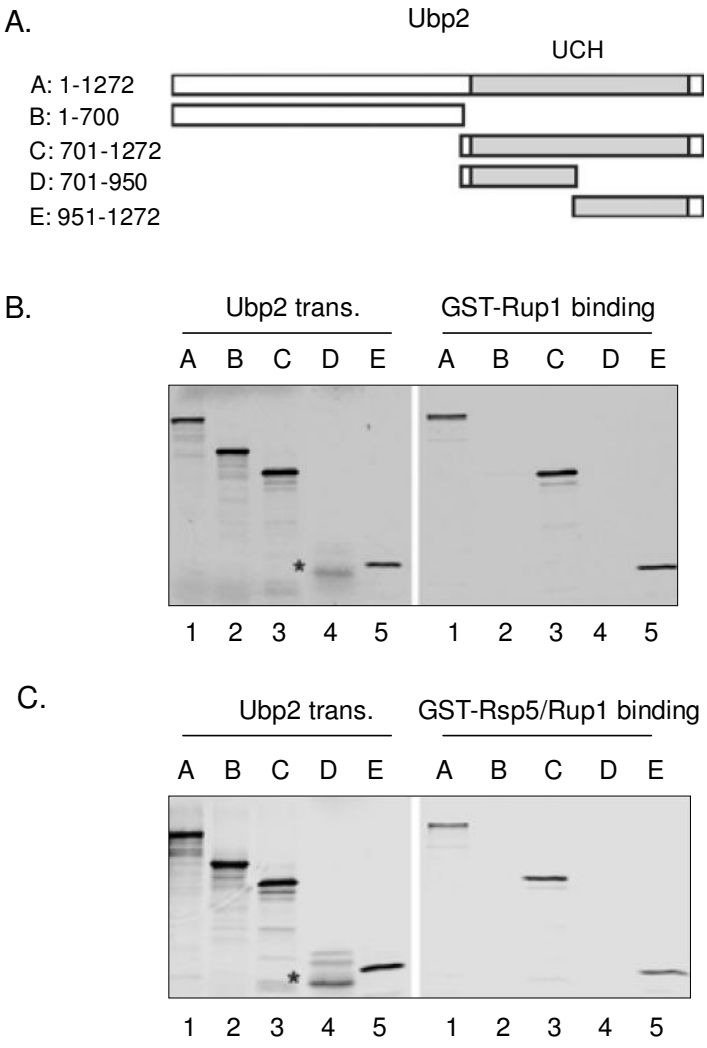


Figure 2.8 Domains of Ubp2 required for binding to Rsp5 and Rup1. (A) Schematic Ubp2 mutants used in binding assays. (B) Binding of GST-Rup1 to Ubp2 proteins. ^{35}S-labeled *in vitro*-translated Ubp2 proteins were incubated with GST-Rup1 and bound

proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel). * indicates predicted size of primary translation product for Ubp2-D. (C) Binding of GST-Rsp5 to Ubp2, in the presence of Rup1. ^{35}S-labeled *in vitro*-translated Ubp2 proteins were incubated with GST-Rsp5 in the presence of added Rup1 protein, and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel).

Genetic interactions between Rsp5, Ubp2, and Rup1

Since Rsp5 and Ubp2 catalyze opposing reactions, we hypothesized that Ubp2 might exist in a complex with Rsp5 in order to antagonize and potentially regulate Rsp5 activity. To test this hypothesis, I overexpressed Ubp2 under control of a galactose-inducible promoter in a wild-type *RSP5* strain (FY56) and in the *rsp5-1* mutant (FW1808). The premise was that overexpression of Ubp2 would cause a reduction in effective Rsp5 activity and therefore mimic rsp5 loss-of-function mutations. The *rsp5-1* mutation is located within the HECT domain (L733S) and impairs enzymatic activity of the purified protein *in vitro* (227) The *rsp5-1* strain (FW1808) grows normally at 30C, but undergoes a rapid non-cell-cycle-specific growth arrest at 37C. A control vector (empty pYES2) and pYES2 expressing wild-type Ubp2 or the active-site mutant of Ubp2 (C745S) were introduced into the rsp5-1 strain and a wild-type RSP5 strain (FY56). A slight growth defect was seen in the RSP5 background when expression of Ubp2 was induced by galactose, and this effect was not seen with the C745S mutant

(Figure 2.9A). This effect was greatly enhanced in the *rsp5-1* background, suggesting that Ubp2 antagonizes Rsp5 activity. While overexpression of Ubp3 or Ubp4 resulted in a slight growth inhibition of the *rsp5-1* strain, a similar effect was seen in the *RSP5* strain (Figure 2.9B), suggesting that this is unlikely to be related to effects on Rsp5 activity. Overexpression of *HA-RUP1* did not inhibit growth of either the *RSP5* or *rsp5-1* strains at 30C (not shown); however, a growth defect was seen upon overexpression of *HA-RUP1* at 34C in the *rsp5-1* strain (Figure 2.9C). Overexpression of *HA-RUP1* deleted of the UBA domain did not elicit a growth defect, suggesting that, while the UBA domain is not necessary for stable ternary complex formation *in vitro*, this domain is important for mediating the antagonistic effect of Ubp2 on Rsp5 *in vivo*. Anti-HA immunoblotting showed that the full-length and ΔUBA proteins were expressed at the same levels (not shown).

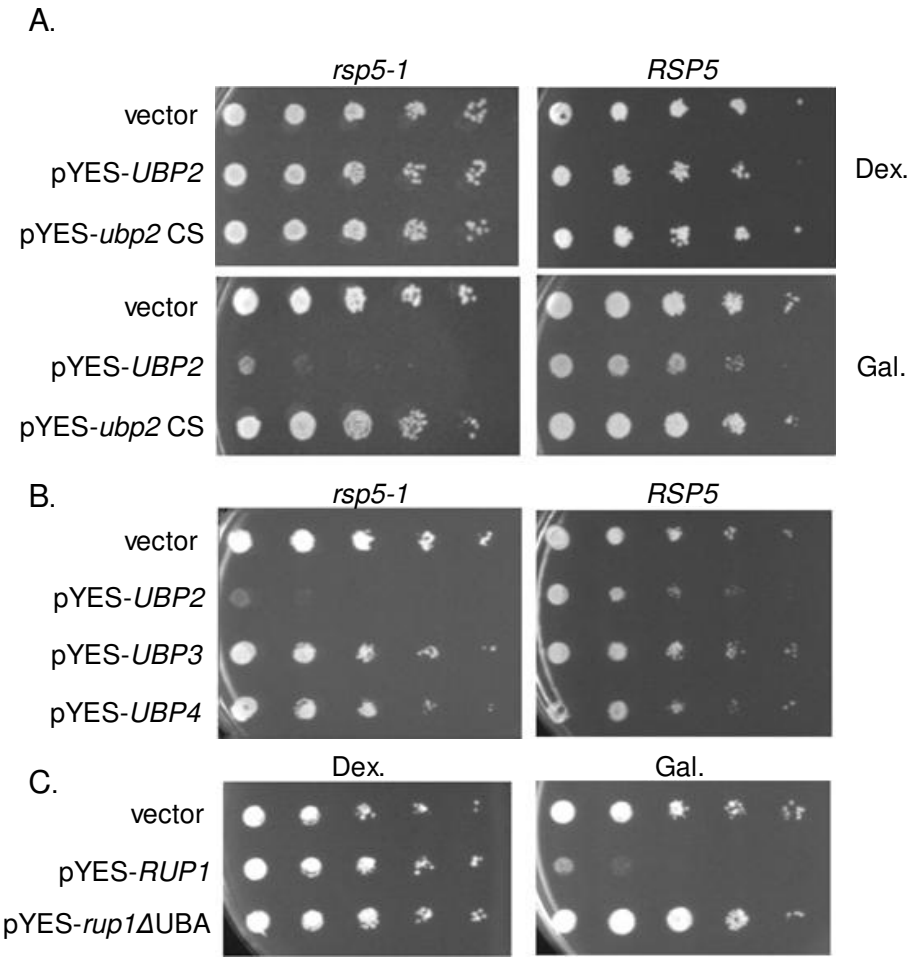


Figure 2.9 Genetic interactions between Rsp5, Ubp2, and Rup1. Overexpression of Ubp2 or Rup1 inhibits growth of the *rsp5-1* mutant. (A) FY56 (*RSP5*) and FW1808 (*rsp5-1*) were transformed with pYES2 (vector), pYES-*UBP2*, or pYES-*ubp2* C745S plasmids. The transformants were serially diluted (10-fold at each step) and plated onto either dextrose (Dex.) or galactose (Gal.) media and grown for 2 and 3 days, respectively, at 30°C. (B) FY56 (*RSP5*) and FW1808 (*rsp5-1*) were transformed with empty vector, or

pYES-*UBP2*, *UBP3*, or *UBP4* plasmids and serially diluted on galactose media and grown for 3 days at 30°C. (C) FW1808 (*rsp5-1*) was transformed with empty vector, pYES-*RUP1*, and pYes-*rup1*ΔUBA plasmids and serial dilutions were plated onto either dextrose or galactose media and grown for 3 and 4 days, respectively, at 34°C.

I further predicted that if *UBP2* and *RUP1* cooperate to antagonize *RSP5* activity, then gene deletion of either *UBP2* or *RUP1* might rescue the temperature sensitivity of the *rsp5-1* mutant by effectively increasing Rsp5 activity. As shown in Figure 2.10, both the *ubp2*Δ and *rup1*Δ mutations partially rescued the temperature sensitivity of the *rsp5-1* mutation at 37C, further suggesting that Ubp2/Rup1 complex is functionally antagonistic to Rsp5. The degree of rescue was more prominent in the case of *ubp2*Δ compared to *rup1*Δ, possibly due to the week interaction between Rsp5 and Ubp2 in the absence of Rup1, as shown in the figure 2.5A.

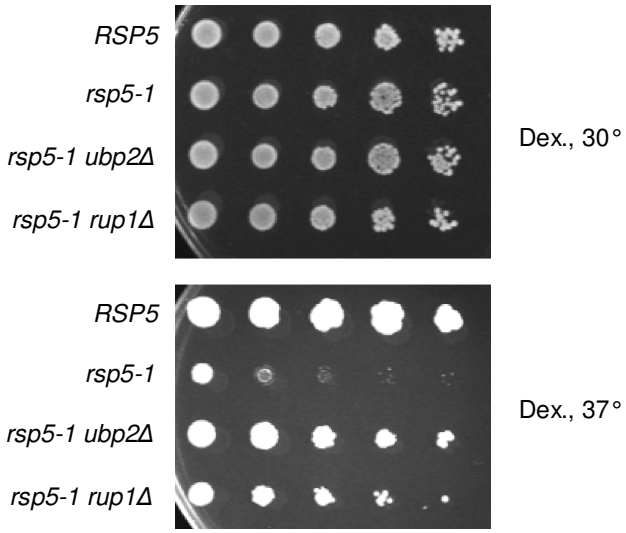


Figure 2.10 Genetic interactions: *ubp2*Δ or *rup1*Δ mutations partially rescue the temperature sensitivity phenotype of the *rsp5-1* mutant. *RSP5* (FY56), *rsp5-1* (FW1808), *rsp5-1, ubp2*Δ (YK003), and *rsp5-1, rup1*Δ (YK004) strains were serially diluted and grown on dextrose-containing media at 30 or 37°C for 2 and 4 days, respectively.

An essential function of Rsp5 at 30C has been shown to be ubiquitin-mediated activation of the Spt23 and Mga2 transcription factors, which are necessary for *OLE1* gene expression and biosynthesis of oleic acid. I therefore tested whether the growth

defect due to Ubp2 overexpression was related to a deficiency in oleic acid biosynthesis by attempting to suppress the *UBP2* overexpression phenotype with exogenous oleic acid. Growth was weakly rescued by oleic acid (Figure 2.11A), suggesting that Ubp2 functions, in part, to antagonize the ability of Rsp5 to activate the Spt23 and/or Mga2 transcription factors. However, the fact that rescue by oleic acid was weak indicates that the Rsp5–Ubp2 enzyme pair affects more than a single aspect of fitness as 30C. The genetic interactions described above indicate that elevated Ubp2 activity, relative to Rsp5 activity, mimics loss of function of Rsp5. I was interested in whether the opposite scenario - elevated Rsp5 activity relative to Ubp2 - resulted in a discernible phenotype. While *S. cerevisiae* requires oleic acid for survival, an excess of oleic acid, through hyperactivation of *OLE1* gene transcription, for example, is also toxic (99, 214). Consistent with this, overexpression of Spt23 from a galactose-inducible promoter resulted in a strong growth defect in a wild-type RSP5 background (Figure 2.11B). Because Rsp5 is required for ubiquitin-mediated activation of Spt23, we predicted that the growth defect due to overexpression of Spt23 would be diminished in the *rsp5-1* background, and this was indeed the case (Figure 2.11B). Furthermore, Spt23 overexpression in the *ubp2*Δ mutant enhanced the toxicity due to Spt23 overexpression in the RSP5 background. These results indicate that elevated Rsp5 activity relative to Ubp2 activity results in hypersensitivity to the effects of Spt23 overexpression and further substantiates an antagonistic relationship between Rsp5 and Ubp2.

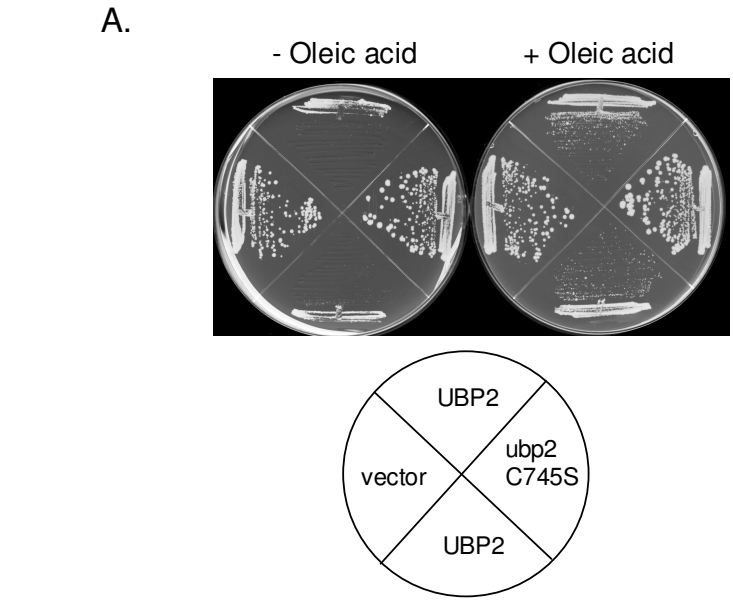


Figure 2.11 Genetic interactions: genetic link between Ubp2 and the OLE pathway (A) Exogenous oleic acid partially suppresses the growth inhibition due to Ubp2 overexpression. FW1808 (*rsp5-1*) transformed with pYES2 (vector), pYES-*UBP2* (in duplicate), or pYES-*ubp2* C745S was streaked onto galactose-containing media, in the absence or presence of added oleic acid, and grown at 30°C for 4 days. (B) Toxicity due to Spt23 overexpression is suppressed by *rsp5-1* and enhanced by *ubp2*Δ. Upper panel:

The pYES-*SPT23* plasmid was transformed into FY56 (*RSP5*) or FW1808 (*rsp5-1*) and plated on galactose- or dextrose-containing media. Lower panel: The empty pYES vector or pYES-*SPT23* plasmid was transformed into either *UBP2*, *ubp2*Δ, or *ubp5*Δ strains and plated onto galactose- or dextrose-containing media and grown for 4 days at 30°C.

Temperature sensitivity of the *rsp5-1* mutant was rescued by the addition of 1M sorbitol to the growth media (Figure 12A), as previously shown for the *rsp5-101* mutant (244), suggesting that ubiquitination of one or more targets of Rsp5 is important for osmotic stability. I therefore predicted that the Ubp2 overexpression phenotype in the *rsp5-1* background at 30C might be due, in part, to osmotic instability. This was confirmed, as shown in Figure 2.12B. The suppression of the *UBP2* overexpression phenotype by 1M sorbitol was significantly more robust than rescue by oleic acid, suggesting that the predominant defect due to overexpression of Ubp2 is related to osmotic instability rather than oleic acid metabolism.

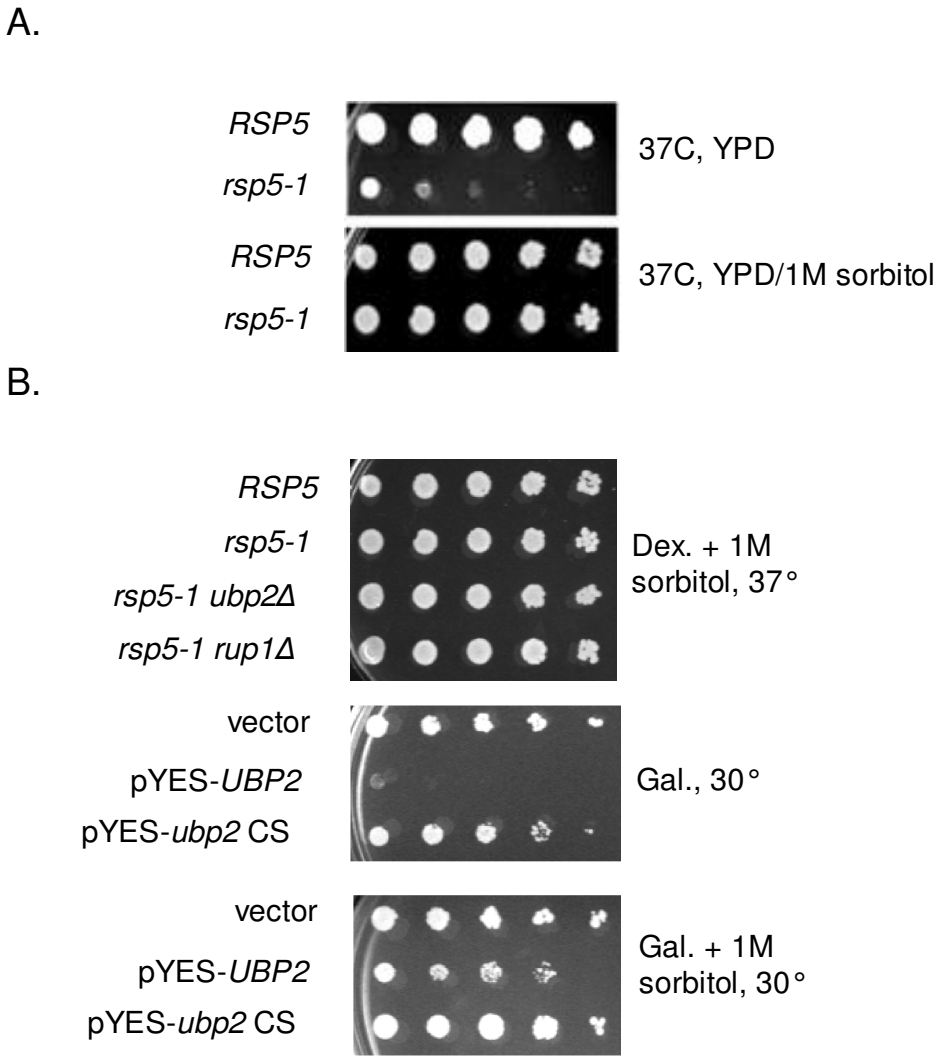


Figure 2.12 Genetic interactions: the *UBP2* overexpression phenotype in the *rsp5-1* mutant is rescued by 1 M sorbitol. (A) *RSP5* (FY56) and *rsp5-1* (FW1808) strains were plated on dextrose-containing media at 37°C, with or without 1 M sorbitol. (B) The indicated FW1808 transformants were serially diluted and plated onto galactose-containing media at 30°C, with or without the addition of 1 M sorbitol to the media. The – sorbitol plate was grown for 3 days, and the + sorbitol plate was grown for 4 days.

Ubp2 reverses Rsp5-catalyzed ubiquitination in vitro

To test whether Ubp2 directly opposes Rsp5 activity *in vitro*, I performed *in vitro* ubiquitination assays with two substrates of Rsp5, Spt23 and WBP2. Spt23 is a biologically relevant Rsp5 substrate, while WBP2 is a human protein that is recognized by Rsp5 and Nedd4, a human homolog of Rsp5 (189). These assays were performed with yeast Ubc1 as the E2 enzyme, which has been shown to function with Rsp5 *in vivo* (49), and mammalian UbcH7 as well as Arabidopsis Ubc8 (not shown). As shown in Figure 13A, Rsp5 efficiently catalyzed polyubiquitination of *in vitro*-translated Spt23 and WBP2. Interesitngly, Ubp2 efficiently deconjugated the pre-ubiquitinated substrates when it was added to the reaction 30 minutes after the ubiquitination reaction. The catalytic active site mutant of Ubp2 (Ubp2C745S) did not have any effect, as expected. Notably, Rup1, which is required for the interaction between Rsp5 and Ubp2, was not required for the deubiquitinating activity (Figure 2.13A lane 3s in both panels). However, if the concentration of Ubp2 is limited, the deubiquitinating activity was partially dependent on the presence of Rup1 (Figure 2.13B). The addition of Rup1, 30 min after initiation of the ubiquitination reaction, did not affect ubiquitination of Spt23 or WBP2. The addition of both Rup1 and Ubp2 resulted in enhanced deubiquitination. Therefore, Ubp2 can reverse Rsp5-catalyzed ubiquitination *in vitro*, and Rup1, while not absolutely required, stimulated this activity of Ubp2.

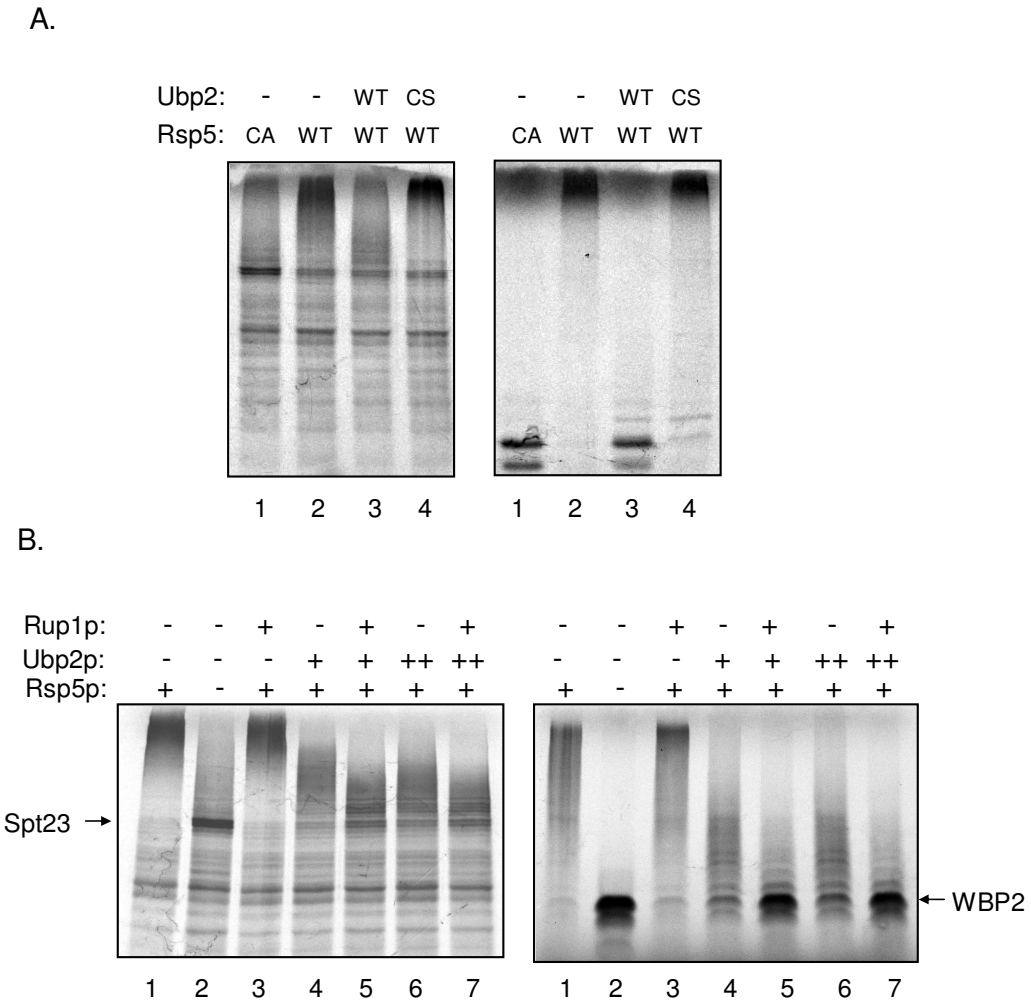
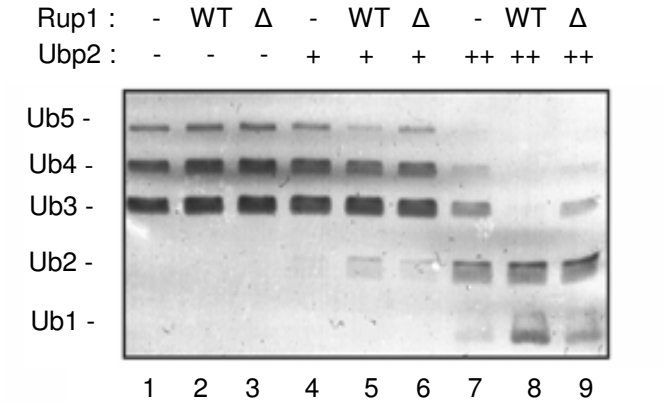


Figure 2.13 Ubp2 antagonizes Rsp5-catalyzed ubiquitination *in vitro*. Rsp5 ubiquitination assay utilized ^{35}S-labeled *in vitro*-translated Spt23 (left) and WBP2 (right). Each reaction contained purified E1, E2 (Ubc1), Ub, ATP in the absence or presence of Rsp5. At 30 minutes after initiating the ubiquitination reaction, Rup1 and/or Ubp2 (two different concentrations) were added, followed by additional 30 minutes incubation. The reactions were stopped by adding 1X SDS-loading buffer and analyzed by 8% SDS-PAGE and visualized by autoradiography.

Figure 2.14 shows that, at the similar range of lower concentrations of Ubp2 as in figure 2.13B, Rup1 enhanced the activity of Ubp2 against free polyubiquitin chains. Interestingly, this effect was dependent on the UBA domain of Rup1, suggesting that the UBA domain plays a role in antagonizing Rsp5 function beyond simply bridging the Rsp5-Ubp2 interaction. This is consistent with the genetic interaction shown in figure 2.9A, where Rup1 overexpression inhibited the growth of the *rsp5-1* mutant, wherease Rup1 deleted of UBA domain did not, suggesting a critical role of the UBA domain in the Ubp2 activitiy. The UBA domain also partially stimulated the deubiquitinating activity when ubiquitin chains were conjugated on to WBP2.

A.



B.

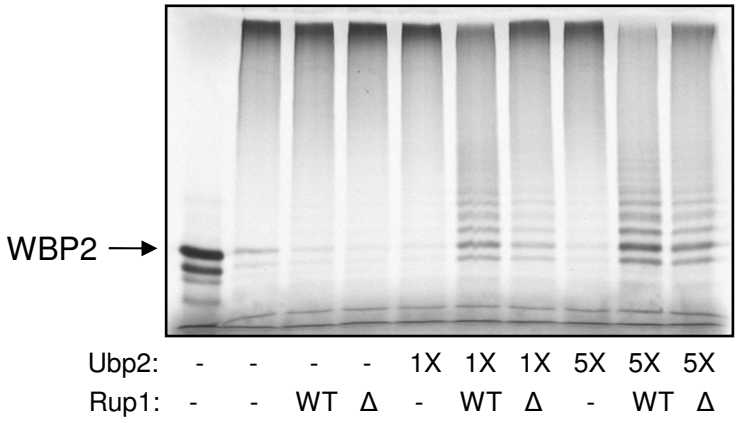


Figure 2.14 UBA domain of Rup1 stimulates Ubp2-catalyzed disassembly of both free K63-linked chains and substrate-linked K63-polyubiquitins. (A) Disassembly of free K63-linked chains were assayed using low concentration of Ubp2 in the presence of no Rup1 (-), Rup1 (WT), and Rup1-ΔUBA (Δ) proteins. After 30 minutes of the deubiquitinating reaction at room temperature, the products were stopped by adding 1X SDS-loading buffer and analyzed by 12% SDS-PAGE followed by Coomassie stain.

(B). Same assays were performed using pre-ubiquitinated ^{35}S-labeled WBP2. After 30 minutes, the products were analyzed by 8% SDS-PAGE followed by autoradiography.

The genetic and biochemical evidence described so far are consistent with the model that Ubp2 antagonizes Rsp5-mediated substrate ubiquitinaiting activities. An important question is whether Ubp2 deubiquitinates only a subset of Rsp5 substrates or all of the substrates. Based on the stoichiometry analysis from the TAP-Rsp5 pulldown, it appears that the ratio of TAP-Rsp5 to Ubp2 might be greater than 1:1 (see figure 2.1), suggesting that not all Rsp5 is in a complex with Ubp2. To better address this issue, gel filtration analysis was performed to see if Ubp2 and Rsp5 move together in same fractions using TAP-UBP2 cell extracts (Figure 2.15). Since it was previously shown that both Rsp5 and Ubp2 bind to DEAE anion exchange column and eluted by high salts (not shown, see figure 2.4A), the cell extracts were first partially purified by DEAE sepharose to increase the purity. Surprisingly, TAP-Ubp2 was present in only few fractions, wherase Rsp5 was distributed into many fractions in the S300 column, suggesting that only a subset of Rsp5 proteins are in complex with Ubp2, and this is consistent with the stoichiometry analysis from the TAP-Rsp5 pulldown. However, it still remains to be determined whether all Rsp5 substrates are subject to deubiquitination by Ubp2 in the cells.

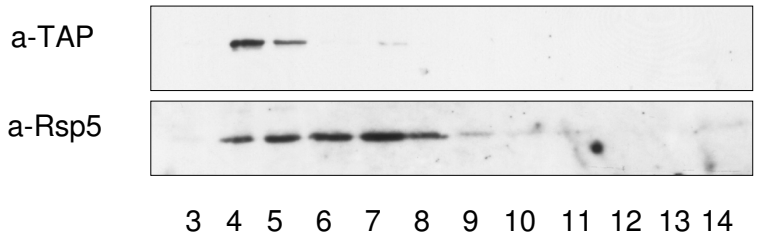


Figure 2.15 Gel filtration analysis of Rsp5/Ubp2 complex. First, cell extracts from 30 ml of TAP-UBP2 (YK005) strain was partially purified on DEAE-sepharose and eluted by 500mM NaCl (see materials and methods). The high-salt eluted fraction was subject to S300 size exclusion column. Equal amounts of fractions were collected and analyzed by 10% SDS-PAGE, followed by western blotting using either anti-Rsp5 or anti-TAP antibodies (for detecting TAP-Ubp2).

Figure 2.13 showed that bacterially purified Ubp2 reverses Rsp5-mediated polyubiquitination of WBP2 and Spt23 *in vitro*. To further test whether Rsp5 is associated with catalytically active Ubp2 from yeast cell extracts, GST-pulldown assay was done using bacterially purified GST-Rsp5 on glutathione sepharose (Figure 2.16).

The GST-Rsp5 was pre-incubated with yeast cell extracts before it was used for deubiquitination assays. The substrate WBP2 was ubiquitinated in separate reactions, prior to the deubiquitination assay. To our expectation, when the pre-incubated GST-Rsp5 was added to the ubiquitinated WBP2, efficient deubiquitination was observed, suggesting that GST-Rsp5 purified active Ubp2 from the yeast cells. Notably, when GST-Rsp5 was pre-incubated with *ubp2*Δ cell extracts, no deubiquitination of WBP2 was detectable, suggesting that the deubiquitination activity purified from wild type cell extracts were due to Ubp2. Furthermore, this suggests that Ubp2 is the only deubiquitinating enzyme associated with Rsp5 in cells. As will be discussed in chapter 5, this type of assay can be used as a test whether the mammalian homologues of Rsp5 (*e.g.*, Nedd4, Smurf1/2, Itch) are associated with DUBs.

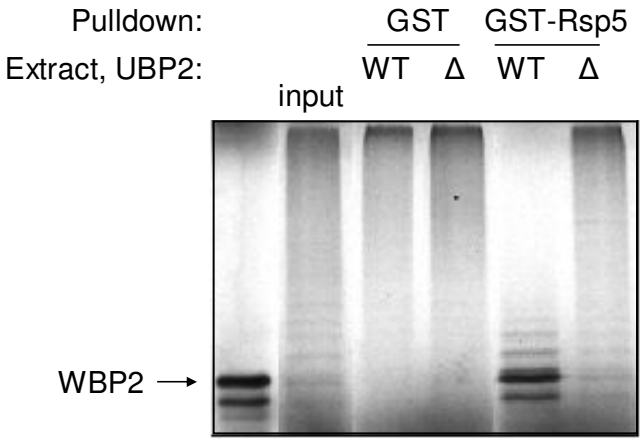


Figure 2.16 GST or GST-Rsp5, on sepharose beads, was used to isolate Ubp2 deubiquitinating activity from cell extract of either WT (*UBP2*) or Δ (*ubp2*Δ). After washing, the beads were incubated with pre-ubiquitinated ^{35}S-labeled WBP2 (lane1, unmodified input; lane 2, pre-ubiquitinated input). Deubiquitination of WBP2 was only seen where GST-Rsp5 had been pre-incubated with wild type cell extract.

2.4 Discussion

The results presented here represent a unique demonstration of the physical coupling of an HECT ubiquitin ligase with a DUB for the purpose of modulating substrate modification. At least four examples of specific interactions between ubiquitin ligases and DUBs have been previously reported. These involve the human Nrdp1 ligase and USP8 (239), the herpes simplex virus ICP0 ligase and USP7 (18), the TRAF2/TRAF6 ligases and CYLD (17, 127, 220), and the VHL ligase and the VDU1 and VDU2 DUBs (138). The ligases in the first three cases are RING domain E3s that undergo autoubiquitination, and in all cases, the DUB has been proposed to reverse the autoubiquitination. In contrast, our results strongly suggest that Ubp2 modulates ubiquitination of Rsp5 substrates, including Spt23, rather than self-ubiquitination of Rsp5. Rsp5 has not been shown to undergo autoubiquitination *in vivo*, consistent with the observations that the half-life of wild-type Rsp5 is similar to that of the active-site cysteine mutant of Rsp5 (227), and overexpression or deletion of Ubp2 does not affect the steady-state level of Rsp5 (not shown). More similar to the Rsp5–Ubp2 relationship, the VHL-associated DUB, VDU2, appears to rescue a VHL substrate (HIF-1a) from degradation (138). In this case, however, VDU2 interacts directly with the substrate, whereas our results suggest that substrate specificity of Ubp2 is likely to be conferred through its Rup1- dependent association with Rsp5.

Rsp5 and Ubp2 only formed a stable complex in the presence of Rup1, and genetic relationships were consistent with the notion that Rup1 cooperates with Ubp2 to antagonize Rsp5 activity. While the UBA domain of Rup1 was not required to mediate

formation of the ternary complex *in vitro*, the UBA domain was necessary for stimulation of Ubp2 activity against free K63 chains at limiting concentrations of Ubp2. It will be of interest to determine whether the Rup1 UBA domain binds polyubiquitin chains, as reported for other UBA domains (24, 179, 233), and whether it interacts preferentially with K63-linked chains. We speculate that the UBA domain might aid in presenting polyubiquitin chains to Ubp2 or, alternatively, might aid in stabilizing an active conformation of Ubp2. The chain specificity of Ubp2 was the same in the absence or presence of Rup1, indicating that this is an inherent characteristic of the enzyme, rather than an Rsp5- or Rup1-dependent effect. A few DUBs have been reported to disassemble both K48- and K63-linked chains *in vitro*, including Cezanne/A20 (59, 232), Ubp14/isopeptidase T (61), and CYLD (127, 220), while AMSH, a JAMM motif isopeptidase, has been shown to have a strong preference for disassembly of K63 chains (157).

While Ubp2 and Rsp5 catalyze opposing reactions, it was conceivable that coupling of Ubp2 to Rsp5 activities might promote, rather than antagonize, at least a subset of Rsp5 functions. For example, Spt23 has been reported to be monoubiquitinated *in vivo* (180). Ubp2 could conceivably promote Rsp5 function by limiting chain extension to favor monoubiquitination. In contrast, there are examples of DUBs that rescue substrates from ubiquitination (25, 110, 139). The observed genetic interactions between *RSP5* and *UBP2* are most consistent with the latter examples, and suggest a model in which Ubp2 rescues Rsp5 substrates from ubiquitination. An antagonistic relationship between Rsp5 and Ubp2 was supported by the observations that (1)

overexpression of *UBP2* in the *rsp5-1* background resulted in a strong growth suppression, (2) *rsp5-1* temperature sensitivity was rescued by either the *ubp2*Δ or *rup1*Δ mutations, and (3) the *ubp2*Δ mutation sensitized cells to the effects of Spt23 overexpression. It is not known whether all functions of Rsp5, or only a subset, are subject to modulation by Ubp2.

There are nine human homologs of Rsp5 (*e.g.,* WWP1/2, Smurf1/2, Nedd4, Itch) involved in various functions, including disease states (Liddle's syndrome, Epstein–Barr virus latency), the life cycle of several budding viruses, and TGFß signaling (reviewed in (110). The results presented here raise the question of whether the activities of these enzymes are also modulated by physically coupled DUBs. The fact that many of the functions of the mammalian Rsp5 homologs are also related to trafficking of membrane proteins suggests that similar regulatory mechanisms might be utilized (see Chapter 5).

CHAPTER THREE

Ubp2 modulates Rsp5-mediated K63-linked polyubiquitination

3.1 Introduction

Ubiquitination of target proteins is catalyzed by a cascade of at least 3 classes of enzymes, known as E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (discussed in detail in chapter 1) (174). There are two main classes of E3 ubiquitin ligases, known as HECT and RING E3s. HECT E3s participate directly in the chemistry of ubiquitin conjugation by forming a covalent thioester intermediate with ubiquitin at an active site cysteine residue within the HECT domain (193), while RING E3s appear to function as docking surfaces for activated E2s and substrates. Rsp5 is the best characterized HECT E3 in *Saccharomyces cerevisiae* and direct homologs of Rsp5 (the Nedd4 family of HECT E3s) exist in all animals (110). *RSP5* is the only essential gene among the five HECT E3s in budding yeast (227), and the minimal essential function of Rsp5 is the ubiquitination of the Spt23 transcription factor, leading to a proteasome-catalyzed processing event that is required for its activation (99). Rsp5 has been reported to function in a variety of other cellular processes including ubiquitin-mediated endocytosis of plasma membrane proteins such as Gap1 (66), Fur4 (206), and Ste2 (50), and the delivery of biosynthetic cargo, such as Cps1, into the endosomal lumen (117). Rsp5 has also been implicated in RNA export (166), ubiquitination of the large subunit of RNA pol II (107), mitochondrial inheritance (64), and cell wall biogenesis (114), indicating that Rsp5 is a multifunctional protein capable of ubiquitinating many substrates in many different locations.

Ubiquitination is a reversible process in that the isopeptide bond between ubiquitin and a substrate protein, as well as isopeptide bonds between ubiquitin

molecules in a polyubiquitin chain, can be cleaved by deubiquitinating enzymes (DUBs). There are at least 18 DUBs characterized in *S. cerevisiae* to date, including 16 ubiquitin specific proteases (UBPs), an ubiquitin C-terminal hydrolase (UCH), and a JAMM motif metalloprotease. The best characterized function of DUBs is to facilitate the rescue of ubiquitin monomers from proteolytic degradation. For example, Doa4/Ubp4 is an endosomal membrane associated protease that cleaves ubiquitin molecules from endocytic cargo prior to vacuolar degradation, thus maintaining total cellular ubiquitin pools (5, 52), and Ubp6 is a proteosome-associated enzyme that is required for ubiquitin homeostasis and thought to rescue ubiquitin molecules prior to proteosomal degradation of substrates (133). Other roles of the DUBs include processing of ubiquitin precursors to mature forms and reversing ubiquitination of substrates (4). There have been several reports of E3s that are physically associated with DUBs (18, 89, 239), and in these cases the DUBs reverse autoubiquitination of the E3s, thus increasing the stability of the E3 by protecting them from proteosomal degradation. Recently, Rsp5 has been shown to be physically associated with Ubp2 (120). Autoubiquitination of Rsp5 *in vivo* has not been previously reported and the half-life of Rsp5 is unaffected by its catalytic activity, suggesting that Ubp2 does not regulate the stability of Rsp5. Rather, genetic and biochemical evidence indicated that Ubp2 antagonizes Rsp5-mediated ubiquitination of target proteins (120). The interaction of Rsp5 and Ubp2 is indirect and is mediated by a third protein, Rup1. Rup1 contains a UBA ubiquitin binding domain and no other characterized functional domains or motifs.

Ubiquitin contains seven lysine residues that can potentially serve as acceptor sites for additional ubiquitin molecules to form polyubiquitin chains. K48-linked polyubiquitin chains are the primary signal for targeting to the 26S proteasome, while monoubiquitination and other forms of polyubiquitin chains appear to mediate alternative functions (21, 63, 91). K63-linked chains serve non-proteolytic functions in DNA repair pathways (94, 208), kinase activation (43), and receptor endocytosis (66, 206), although they may also be capable of proteasome targeting (187). K29-linked chains were reported to be a degradation signal and recently shown to lead to an endocytic signal (20, 111), and K6-linked chains have been implicated in a DNA repair pathway (237). The functions of polyubiquitin chain types are summarized in Illustration 3.1.

In this chapter, I show that Rsp5 preferentially catalyzes K63-linked polyubiquitination of substrates *in vitro*, while Ubp2 preferentially disassembles K63 chains *in vitro.* Furthermore, Rsp5 and Ubp2 modulate a significant fraction of K63-linked polyubiquitination *in vivo*, and the essential function of Rsp5 at elevated temperature requires K63 polyubiquitination. These findings will provide excellent tools for identification and characterization of biochemical mechanisms for the synthesis of K63-linked polyubiquitination as well as the conjugated substrates.

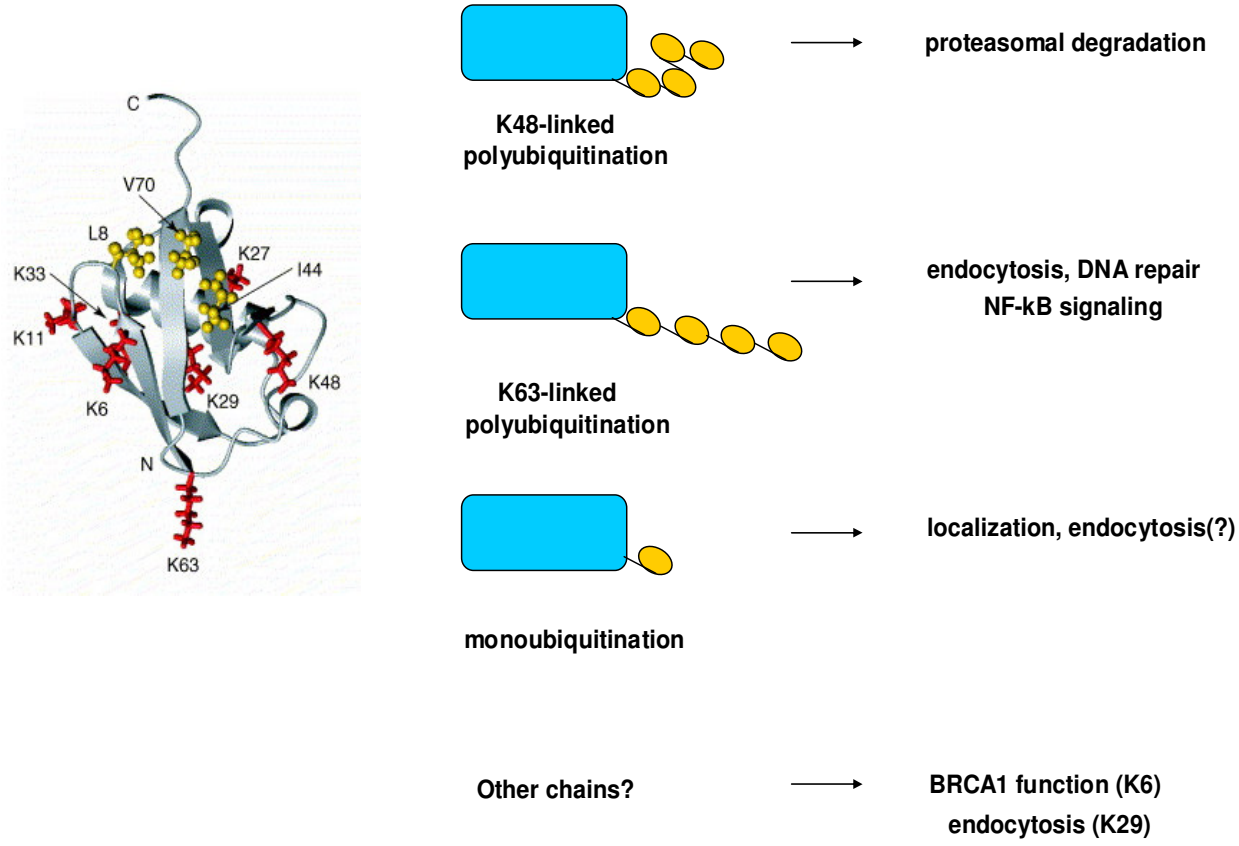


Illustration 3.1 Functions of different polyubiquitin chain types. Left. Relevent features of ubiquitin. The seven lysine residues are shown in red, while the L8–I44–V70 hydrophobic patch is shown in gold ball-and-stick. Reprinted from Pickart and Fushman (175). Copyright (2004), with permission from Elsevier. Right. Characterized roles of each chain types. See text.

3.2 Materials and methods

Yeast strains, media, and plasmids

A list of yeast strains is shown in Table 1. SUB492 and SUB493 strains were generous gifts from Dr. Dan Finley and express wild type and K63R ubiquitin as a sole source of ubiquitin, respectively, as previously described (208) (see table 3.1). *ubp2*Δ mutations were introduced into both SUB492 and SUB493 strains using kanamycin (KanMX6) selection, as previously described (120) (see chapter 2), generating YK018 and YK019, respectively. pUB39 is a Lys2-marked plasmid that expresses wild type ubiquitin under the *CUP1* promoter. pUB115, pUB192, pUB195, and pUB197 are identical to pUB39, except for the K48R, K6R, K29R, and K63R mutations in the ubiquitin, respectively (208), and were generous gifts from Dr. Dan Finley. Plasmids for overexpressing *UBP2* and *ubp2*C745S were described previously in chapter 2. For the ADCB containing media, ADCB (Sigma) was dissolved in water and added to synthetic minimal media as final concentrations of 100 or 200ug/ml where indicated. For the CFW containing media, CFW (Sigma) was dissolved in water and added to YPD media as final concentrations of 5 or 7ug/ml where indicated.

Protein interaction assays

GST-fusions of Rsp5 and Ubp2C-S were expressed from the pGEX6p-1 (Amersham Biosciences) in *Escherichia.coli* DH5α strain by standard methods and affinity-purified on glutathione-Sepharose (Amersham Biosciences). The purified proteins were cleaved from GST using PreScission protease (Amersham Biosciences)

under the manufacturer's recommended conditions. For the pulldown of ubiquitin conjugates from the wild type (BY4741) and *ubp2*Δ (YK009) cell extracts, cells in mid-log phase (typically 30 O.D) were lysed with a bead beater in NP40 lysis buffer (1% NP40, 150mM NaCl, 50mM Tris pH 7.0) in the presence of protease inhibitors (leupeptin, aprotinin, PMSF). Cell extracts were cleared by centrifugation at 14,000 rpm for 10 minutes before they were subject to GST-Rsp5 or GST-Ubp2C-S sepharose. The binding reactions were left for 2 hours at 4°C and the beads were washed with the NP40 buffer for 3 times before analyzing by 8% SDS-PAGE. The gels were electro-blotted onto nitrocellulose membranes and western blot analyses were performed using monoclonal anti-ubiquitin antibody (Santacruz Biotech.)

In vitro ubiquitination/deubiquitination assays

In vitro ubiquitination and deubiquitination assays were performed in the presence of 10mM Tris pH 7.5, 50mM NaCl, 5mM ATP, 5mM $MgCl_2$, 0.1mM DTT, and 50ug/ml ubiquitin (Sigma). Bacterially expressed Rsp5, Rup1, and Ubp2 were purified on glutathione sepharose and GST was removed by cleavage with PreScission protease (Amersham Biosciences). Human E1, UbcH7, E6AP, and HPV16 E6 proteins were expressed in High5 insect cells (Invitrogen) using recombinant baculoviruses. Cell lysates were made 48-hours post-infection in NP-40 lysis buffer (1% NP40, 100 mM Tris, pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT), affinity-purified on glutathione-Sepharose, and cleaved from GST with PreScission protease. *In vitro* translated ^{35}S-labeled p53, Scribble, WBP2, synthesized using transcription and

translation coupled (TNT) rabbit reticulocyte lysate (Promega), were used as substrates. The ubiquitination reactions were performed as previously described in chapter 2. Ubiquitination reactions were carried out for 30 min at room temperature, followed by an additional 30 min for deubiquitination by Rup1/Ubp2. For the assays using K0, K48-only, K63-only ubiquitin (Boston Biochem), the *in vitro* translated substrates were partially purified by DEAE anion exchange column to remove endogenous ubiquitins. Briefly, the *in vitro* translation mixtures were prepared in 50-100ul of volumes. The lysates were diluted 3-4 fold with the ion exchange binding buffer (50mM Tris pH7.0, 20mM NaCl), supplemented with DTT of final 10mM concentration to cleave thioester-bound ubiquitins from E1 and E2s. 30ul of DEAE sepharose (Amersham Biosciences) equilibrated with the same buffer was added to the diluted lysates and incubated for 20 minutes at 4C. The beads were harvested at 2,500 rpm and the supernatant was removed, followed by 3 times of extensive washing with the same buffer. The bound proteins were eluted by adding 50-100ul of 500mM NaCl for 10 minutes. The ubiquitination reactions were performed as described in chapter 2 and the reactions were stopped by addition of SDS-PAGE loading buffer and the samples were analyzed on 8% SDS-PAGE gel, followed by autoradiography. The deubiquitination assays using free ubiquitin chains utilized 3ugs of either K48 or K63 polyubiquitin chains 3-7 (Boston Biochem), and were incubated with 0.15–15 ng of purified Ubp2 for 1 h at room temperature in buffer containing 10mM Tris pH 7.5, 50mM NaCl, 5mM MgCl2, and 0.1mM DTT. The reactions were stopped by adding 1X SDS–PAGE loading buffer and products were

analyzed by 12% SDS–PAGE and staining with Coomassie blue for visualization of the chains.

Strain	Genotype	References
FY56	MATα *his4-912δR5 lys2-128δ ura3-52*	(107)
YK001	MATα *NTAP-RSP5 his4-912δR5 lys2-128δ ura3-52*	(120)
FW1808	MATα *rsp5-1 his4912δR5 lys2-128δ ura3-52*	(107)
YK003	MATα *rsp5-1 ubp2::KANMX6 his4912δR5 lys2-128δ ura3-52*	(120)
YK004	MATα *rsp5-1 rup1::KANMX6 his4912δR5 lys2-128δ ura3-52*	(120)
BY4741	MATa *his3 leu2 met15 ura3*	Open Biosystems
YK009	MATa *ubp2*Δ::*KanMX6 his3 leu2 met15 ura3*	Open Biosystems
SUB492	MATa *lys2-801 leu2-3,112 ura3-52 his3-Δ 200 trp1-1[am] ubi1-Δ 1::TRP1 ubi2-Δ 2::ura3 ubi3-Δ ub-2 ubi4-Δ 2::LEU2* [pUB39][pUB100]	(208)
SUB493	Isogenic to SUB492 except for pUB197 instead of pUB39	(208)
YK018	MATa *ubp2Δ*::KanMX6 *lys2-801 leu2-3,112 ura3-52 his3-Δ 200 trp1-1[am] ubi1-Δ 1::TRP1 ubi2-Δ 2::ura3 ubi3-Δ ub-2 ubi4-Δ 2::LEU2* [pUB39][pUB100]	(121)
YK019	Isogenic to YK018 except for pUB197 instead of pUB39	(121)

Table 3.1 List of the yeast strains used in chapter 3.

3.3 Results

Rsp5 and Ubp2 assembles and disassembles K63-linked polyubiquitin chains in vitro

In chapter 2, it was shown that Ubp2 disassembles at least two substrates of Rsp5, prompting a next question whether Ubp2 can disassemble any kind of polyubiquitinated proteins. To further investigate the biochemical activity of Ubp2 *in vitro*, it was tested if Ubp2 can deubiquitinate the E6/E6AP-catalyzed polyubiquitin chains on two substrates, p53 and Scribble (Figure 3.1). Surprisingly, Ubp2 did not deubiquitinate the E6AP-catalyzed polyubiuitin chains under the same condition where it efficiently deubiquitinated Rsp5 substrates (Figure 2.13 in chapter 2). Two possible mechanisms may explain the discriminatory activity of Ubp2. First, the molecular interaction between Rsp5 and Ubp2 might be critical for the deubiquitinating activity. Second, the type of chains synthesized by Rsp5 and E6AP-catalyzed reactions might be different, leading to differential disassembly by Ubp2. In fact, it was known previously that E6AP catalyzes almost exclusively K48-linked chains *in vitro* (140), while it was shown that at least some of the natural substrates of Rsp5 are modified by K63-linked polyubiquitin chains *in vivo* (52, 64, 66, 206). I therefore investigated the relative preference of Rsp5 and Ubp2 for K63 chain assembly and disassembly, respectively. I performed ubiquitination assays in the presence of ubiquitin in which all lysine residues were altered to arginine except for lysine 48 (K48-only ubiquitin) or lysine 63 (K63-only ubiquitin).

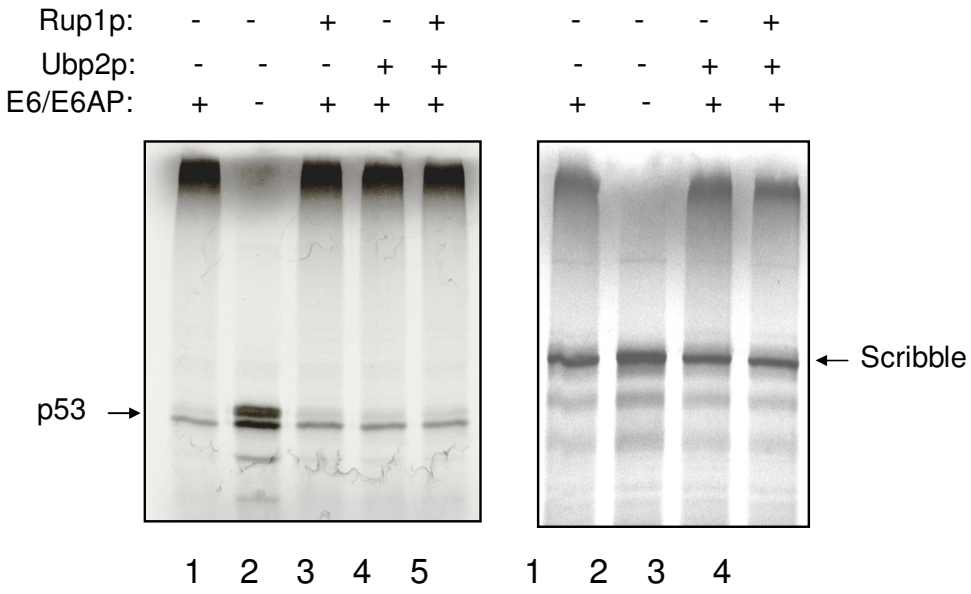


Figure 3.1 Ubp2 does not reverse E6/E6AP-catalyzed ubiquitination reaction *in vitro*. E6AP-mediated ubiquitination of ^{35}S-labeled p53 (left) and Scribble (right) was performed in the presence of E6, E1, E2 (UbcH7), Ub, ATP. After 30 minutes of the ubiquitination reaction, Rup1 and/or Ubp2 were added and incubated for additional 30 minutes, as described previously. The reactions were stopped by adding 1X SDS-loading buffer and the total products were analyzed by 8% SDS-PAGE and visualized by autoradiography.

The endogenous ubiquitin present in the *in vitro* translation reactions was first depleted by anion exchange chromatography (DEAE), and as expected, there was no detectable ubiquitination in the absence of added ubiquitin (Figure 3.2). The addition of wildtype ubiquitin, K48-only ubiquitin, or K63-only ubiquitin resulted in a similar degree of overall substrate modification (i.e., the amount of remaining unmodified substrate was similar; lanes 3–5). However, the average length of the polyubiquitin chains was shorter in the presence of K48-only ubiquitin relative to wild-type ubiquitin, while the average chain length was longer in the presence of K63-only ubiquitin. This is the first demonstration that Rsp5 preferentially synthesizes K63-linked polyubiquitins *in vitro*, consistent with its reported role in the endocytosis of membrane proteins (66, 206). When Ubp2 was added in the reactions, the extent of deubiquitination was similar in the reactions containing wild-type and K63-only ubiquitin, while there was significantly less deubiquitination seen in the K48-only reaction. Experiments with ubiquitin in which all lysines were mutated to arginines suggested that much of the multiubiquitination observed with K48-only ubiquitin was actually the result of monoubiquitination at multiple lysines of the substrate (not shown). Together, these results suggest that Rsp5 preferentially assembles K63-linked chains, while Ubp2 preferentially disassembles K63 chains. Similar experiments with E6/E6AP-catalyzed reactions confirmed that E6AP catalyzed almost exclusively K48-linked chains to both p53 and Scribble, and again, Ubp2 did not disassemble these conjugates (not shown).

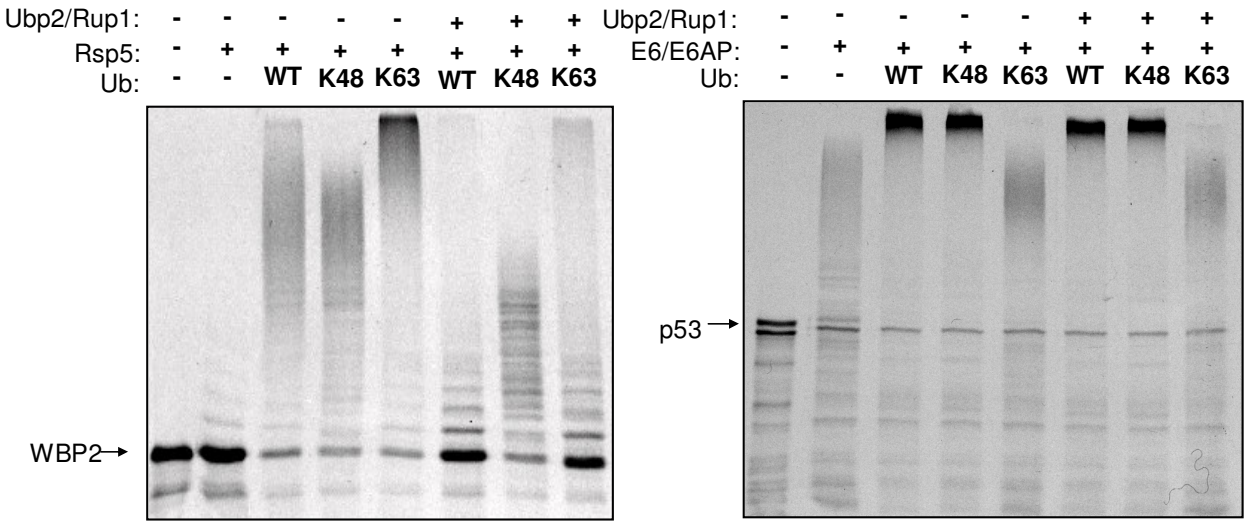


Figure 3.2 Rsp5 and Ubp2 preferentially assemble and disassemble K63-linked polyubiquitin chains. (A) Rsp5-catalyzed ubiquitination reactions were carried out with ^{35}S-labeled WBP2 as a substrate, as in Figure 13 of chapter 2, except that the endogenous ubiquitin present in the translation reaction was first removed by DEAE anion exchange chromatography. The reactions were then performed in the absence of added ubiquitin (left panel, lane 2) or the presence of wild-type ubiquitin, K48-only ubiquitin, or K63-only ubiquitin (left panel, lanes 3–5). Deubiquitination (lanes 6–8) was initiated after 30 min by the addition of Ubp2 and Rup1. Control reactions (lanes 9–11) show that the ubiquitination reaction was dependent on added E2 (Ubc1) and Rsp5. (B) E6/E6AP-catalyzed ubiquitination reactions were performed as in (A). The products were stopped by adding 1X SDS-loading buffer, analyzed by 8% SDS-PAGE, followed by autoradiography.

To confirm the apparent preference of Ubp2 for K63-linked chains, deubiquitination assays were carried out using free purified K63 or K48 chains as substrates over a 100-fold range of Ubp2 concentration. As shown in Figure 3.3, at equivalent enzyme concentrations, Ubp2 showed a strong preference for hydrolysis of K63-linked chains, although there was reactivity against K48-linked chains at higher concentrations of Ubp2. This suggests that the inherent preference of Ubp2 for K63-linked polyubiquitin chains is the likely basis for its capability to deubiquitinate the Rsp5-catalyzed polyubiquitination.

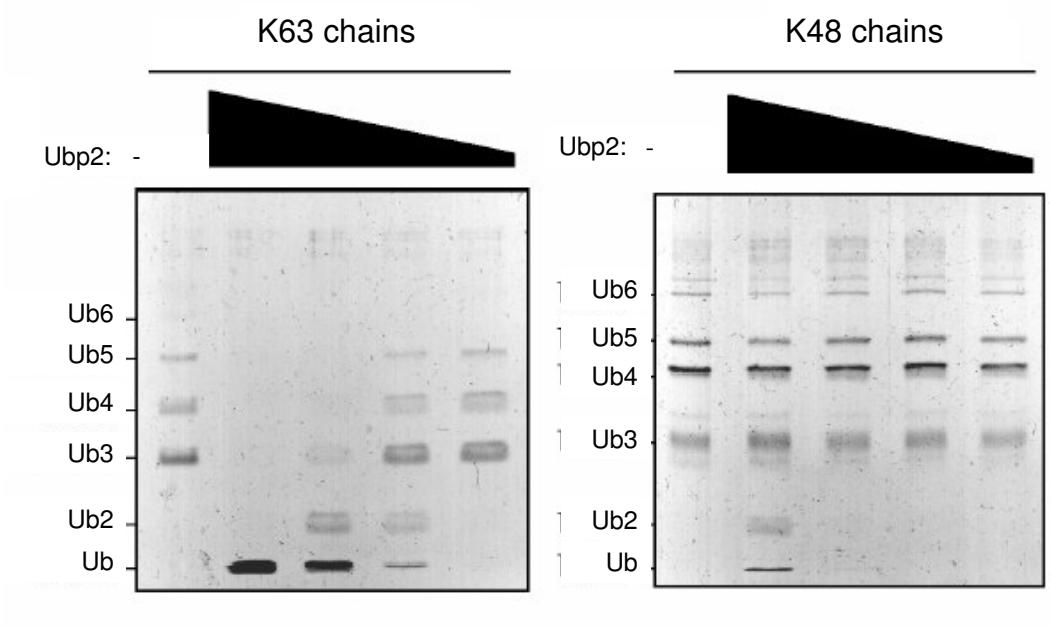


Figure 3.3 Ubp2 preferentially disassembles K63-linked free polyubiquitin chains. Purified K48 and K63-linked polyubiquitin chains (Ub_3-Ub_7; Boston Biochem) were assayed as substrates of purified Ubp2 protein over 100 fold range of Ubp2 concentration. The deubiquitination reactions were performed for 30 minutes at room temperature and the reactions were stopped by 1X SDS-loading buffer. The samples were analyzed by 12% SDS-PAGE followed by Coomassie stain.

Modulation of K63 polyubiquitin conjugates by Rsp5 and Ubp2 in vivo.

To further analyze the Ubp2 activity *in vivo*, I compared the amount of overall ubiquitin conjugates in total cell extracts from wild type *UBP2* and *ubp2Δ* cells. Unexpectedly, a strong increase in total ubiquitin conjugates was seen in *ubp2Δ* cells compared to the wild type cell extracts, as determined by immunoblotting with anti-ubiquitin antibody (Figure 3.4A), suggesting that the increased ubiqutitin-conjugates are likely to be the physiologic substrates of Ubp2. To confirm this, a plasmid-based *UBP2* gene under *GAL1* promoter control was reintroduced into the *ubp2*Δ cells (Figure 3.4B). Consistent with the result in figure 3.4A, the exogenous UBP2 overexpression reduced the conjugate level below that seen in the wild-type *UBP2* strain, while reintroduction of the active-site cysteine-to-serine *ubp2* mutant (C745S; C-S) did not suppress conjugate accumulation.

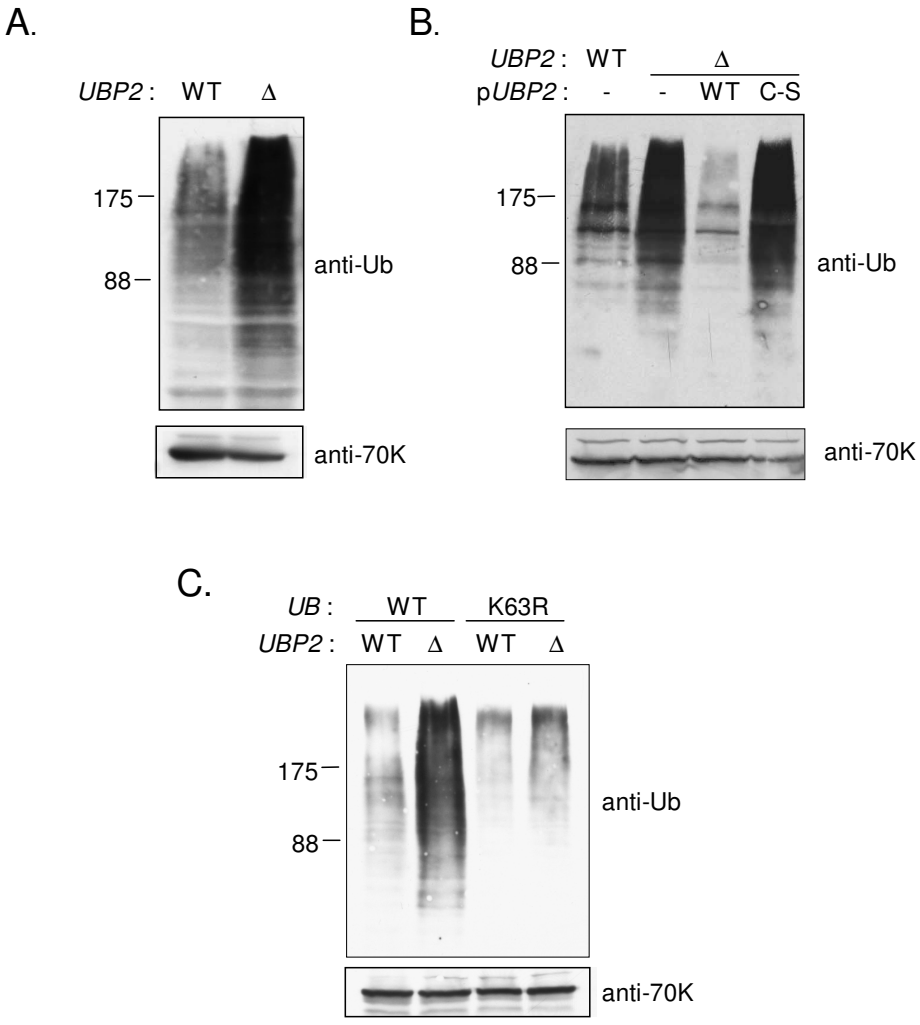


Figure 3.4 Ubp2 modulates K63-linked conjugates in *S. cerevisiae*. (A) Total ubiquitin conjugates were compared in total extracts of *UBP2* (BY4741) and *ubp2Δ* (YK009) cells by immunoblotting with anti-ubiquitin antibody. An anti-Rpa1 immunoblot (anti-70K) is shown of the same extracts as a loading control. (B) Total ubiquitin conjugates in the *ubp2Δ* mutant were examined as in A. upon overexpression of wild type Ubp2 or the catalytically inactive Ubp2-C745S (C-S) proteins. (C) Total ubiquitin conjugates in cell extracts from wild type ubiquitin (SUB492), wild type ubiquitin/*ubp2Δ* (YK018),

K63R ubiquitin (SUB493), and K63R ubiquitin/*ubp2Δ* (YK019) strains were compared by anti-ubiquitin immunoblotting.

Based on the *in vitro* activities of Ubp2 in figure 3.2 and 3.3, it is likely that the increased ubiquitin conjugates in the *ubp2*Δ mutants represent accumulation of K63-linked polyubiquitin chains. To test this, I took advantage of the strains in which all four endogenous ubiquitin genes were eliminated and replaced by a plasmid-borne wild-type ubiquitin gene, or a mutated ubiquitin gene encoding K63R ubiquitin (208). The expressions of exogenous ubiquitin genes are under control of the copper-inducible *CUP1* promoter, and it was shown that the basal non-induced level of the promoter activity result in approximately similar levels of ubiquitins in the cells compared to the sum of all four endogenous ubiquitins. The *ubp2*Δ mutations were introduced into both of these strains and it was confirmed that the growth of the resultant strains were all normal compared to the wild type *UBP2* strains (not shown). Consistent with figure 4A, the *ubp2*Δ mutation led to an increase in ubiquitin conjugates in the strain expressing wild type ubiquitin (Figure 3.4C). Interestingly, although expected, the *ubp2*Δ mutation in the K63R strain resulted in only a slight accumulation of conjugates, strongly suggesting that the vast majority of the elevated ubiquitin conjugates in the *ubp2*Δ mutant represented K63-linked chains. However, the small but consistently increased level of ubiquitin conjugates were observed in the *ubp2*Δ /K63R strain, suggesting that Ubp2 may have a

limited capacity to recognize polyubiquitin linkages other than K63, or possibly monoubiquitins (lane 4 of figure 3.4C).

The *ubp2*Δ mutation conferred increased sensitivity to the toxic proline analogue L-azetidine-2-carboxylic acid (ADCB; Figure 3.5A) (182), suggesting that hyper-K63 polyubiquitination of one or more proteins leads to ADCB hypersensitivity. Consistent with this, the *ubp2*Δ mutant did not display increased ADCB sensitivity in cells that expressed K63R-ubiquitin as the sole source of ubiquitin (Figure 5A, upper left panel). Even in a wild-type *UBP2* background, cells expressing K63R ubiquitin as the sole source of the ubiquitin were resistant to ADCB compared to the wild type ubiquitin strain, further indicating that K63-linked polyubiquitination is required to confer sensitivity to ADCB (Figure 3.5A, lower left upper). *GAL1* promoter-driven expression of *UBP2* in the *ubp2*Δ mutant conferred ADCB resistance beyond that of wild-type *UBP2* cells, while overexpression of the C-S mutant did not suppress ADCB sensitivity (Figure 3.5B).

Ren et al (182) showed that in *ubp2*Δ or *rup1*Δ cells, GFP-Gap1 general amino acid permease is stabilized at the plasmamembrane even in a nitrogen-replete condition, suggesting that Ubp2 and Rup1 are required for the receptor endocytosis, presumably by promoting Rsp5 activity.

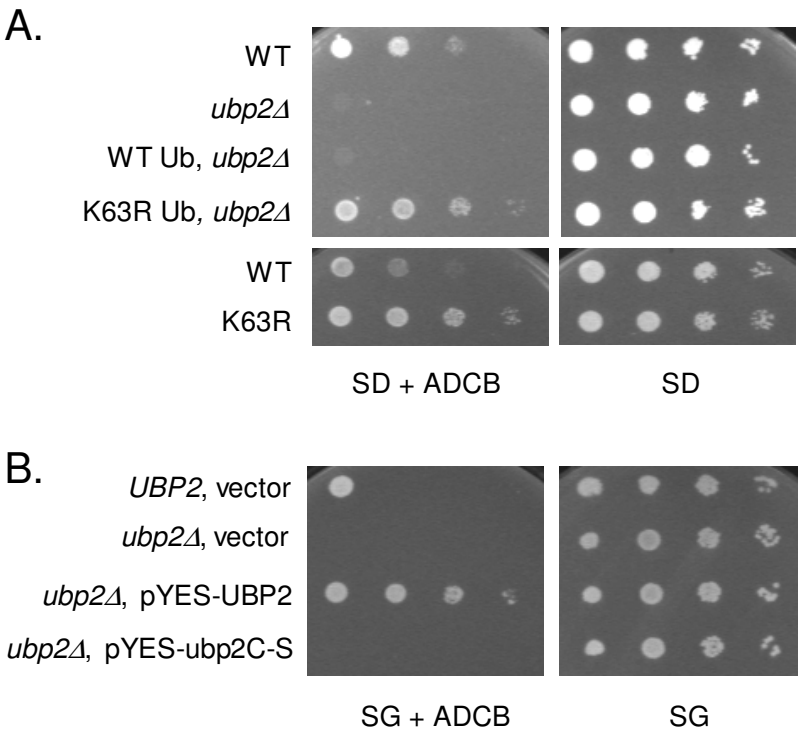
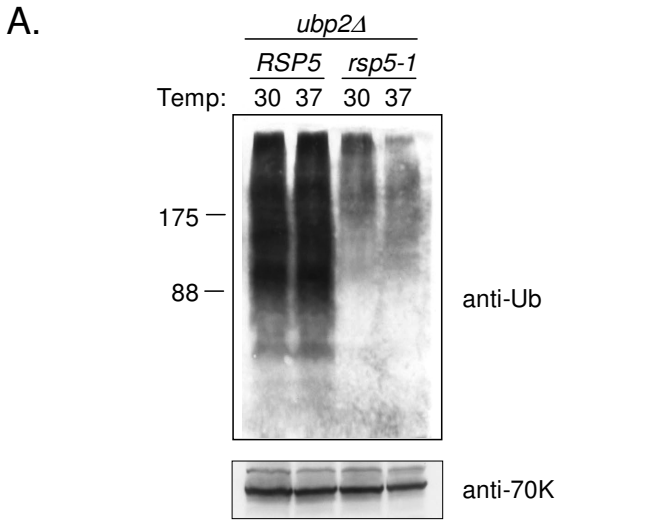


Figure 3.5 ADCB sensitivity of the *ubp2Δ* mutant. (A) Top: *UBP2* (BY4741), *ubp2*Δ (YK009), wild type ubiquitin/*ubp2Δ* (YK018), and K63R ubiquitin/*ubp2Δ* (YK019) cells were 10-fold serially diluted and spotted on either synthetic minimal (SD) media or SD containing 100ug/ml of ADCB and grown at 30°C for 3 days. Bottom: Wild type ubiquitin (SUB492) and K63R ubiquitin (SUB493) cells were 10-fold serially diluted and spotted on either synthetic minimal (SD) media or SD containing 100ug/ml of ADCB and grown at 30°C for 3 days. (B) UBP2 overexpression increases resistance of cells to ADCB. The indicated strains were 10-fold serially diluted and spotted on either synthetic media containing galactose with or without 200ug/ml of ADCB and grown at 30°C for 4 days.

Together, the results shown in Figure 3.4 and 3.5 indicate that the *ubp2Δ* mutation leads to an overall accumulation of K63-linked polyubiquitin conjugates *in vivo*. To determine whether the conjugates that accumulate are the result of Rsp5 ubiquitination activity, I analyzed the effect of the *rsp5-1* hypomorphic temperature-sensitive mutation on the accumulation of conjugates. A strong reduction in overall conjugates was observed in the *rsp5-1/ubp2*Δ mutant compared to *RSP5/ubp2*Δ cells (Figure 3.6A), both at normal and elevated growth temperatures. This result strongly suggests that the majority of ubiquitin conjugates that accumulate in the *ubp2*Δ strain are the result of Rsp5-dependent ubiquitin conjugation, consistent with the previous demonstration that Rsp5 preferentially catalyzes conjugation of K63-linked chains *in vitro* (Figure 3.2).

To further demonstrate that Rsp5 catalyzes K63 chain formation *in vivo*, wild-type ubiquitin and K63R ubiquitin were overexpressed in the *rsp5-1* mutant (Figure 3.6B, top). It was previously reported that overexpression of ubiquitin can rescue the temperature sensitivity of *rsp5-1* strain (129). The premise was that if K63-linked polyubiquitin chain formation is critical for the Rsp5 activity, overexpression of K63R ubiquitin should not rescue the hypomorphic *rsp5-1* strain. Consistent with the previous report, overexpression of wild type ubiquitin under the *CUP1* promoter rescued growth defect of the *rsp5-1* mutant at elevated temperature (35C), although I could not recapitulate the rescue at 37C, at as it was reported (not shown). Interestingly, overexpression of K63R ubiquitin did not rescue the growth defect, suggesting that K63-linked polyubiquitin chain formation is critical for the Rsp5 activity at the elevated temperature. This is consistent with previous observation that growth of the K63R is

severely inhibited at 37C (208). Overexpression of the ubiquitins did not affect the growth of wild type RSP5 strain at both 30C and 37C (Figure 3.6B lower panels). To further investigate the chain type requirement for rescue, K6R, K29R, or K48R ubiquitin were overexpressed in the mutant (Figure 3.6C). As expected, overexpression of K6R and K48R ubiquitins rescued *rsp5-1* temperature sensitivity, suggesting that neither formation of K48 or K6-linked chains are essential activity of Rsp5 at highy temperature. Interestingly, rescue by K29R ubiquitin was only partial compared to the wild type ubiquitin. These results demonstrate that the ability of Rsp5 to form K63-linked conjugates is linked to its essential function at elevated temperature, although they do not rule out that Rsp5 might synthesize other types of polyubiquitin chains *in vivo*, particularly K29-linked chains.


A.
ubp2Δ
RSP5
rsp5-1
Temp:
30 37 30 37
175
88
anti-Ub
anti-70K

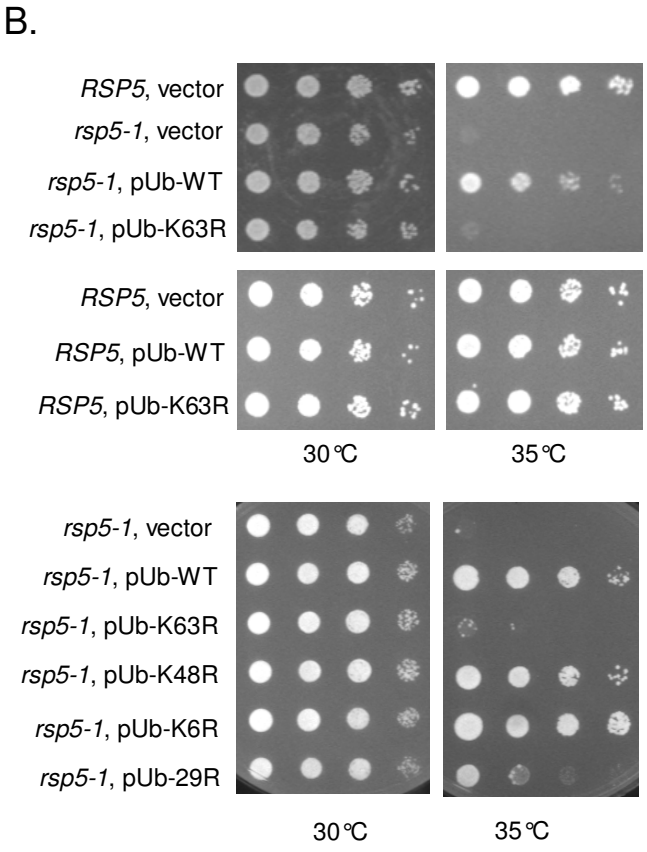

B.
RSP5, vector
rsp5-1, vector
rsp5-1, pUb-WT
rsp5-1, pUb-K63R
RSP5, vector
RSP5, pUb-WT
RSP5, pUb-K63R
30℃
35℃
rsp5-1, vector
rsp5-1, pUb-WT
rsp5-1, pUb-K63R
rsp5-1, pUb-K48R
rsp5-1, pUb-K6R
rsp5-1, pUb-29R
30℃
35℃

Figure 3.6 Rsp5 catalyzes K63-linked polyubiquitination *in vivo*. A. Total ubiquitin conjugates were compared in cell extracts from *ubp2*Δ (YK009) and *rsp5-1/ubp2*Δ (YK003) strains by anti-ubiquitin immunoblotting. B. *rsp5-1* (FW1808) cells transformed with empty plasmid, plasmids expressing wild type ubiquitin (pUb-WT; pUB39), K63R ubiquitin (pUB-K63R; pUB197), K48R ubiquitin (pUB-K48R; pUB115), K6R ubiquitin (pUB-K6R; pUB192), or K29R ubiquitin (pUB-K29R; pUB195) under *CUP1* promoter control were 10-fold serially diluted and plated on minimal media and grown at either 30°C or 35°C for 3 days. Note that exogenous copper was not added in the media.

*Rsp5 and Ubp2 regulate cell wall homeostasis***.**

The temperature sensitivity of *rsp5* mutants can be partially rescued by sorbitol, an osmotic stabilizer (120, 244), and a recent report also indicated that Rsp5 affects cell wall integrity (114). An assay that reflects the effect of *rsp5* mutations on cell wall integrity is sensitivity to calcofluor white (CFW), a chitin binding molecule. Changes in the sensitivity to this drug are generally thought to be indicative of changes in the cell wall structure and/or chitin level (114). As shown in Figure 3.7A, the *rsp5-1* mutant was sensitive to CFW at 30°C, consistent with the previous report in Kaminska et al. According to the model that Rup1 and Ubp2 functionally antagonize Rsp5, we predicted that *ubp2* or *rup1* mutations would lead to increased Rsp5 activity and therefore rescue CFW sensitivity of the *rsp5-1* mutant. Indeed this was the case (Figure 3.7A), indicating that Rup1/Ubp2 activity and Rsp5 activities are balanced, in part, to

achieve cell wall homeostasis. However, it is not clear whether this reflects differences in chitin level of the cell wall among the mutants, as CFW staining of the yeast cells did not reveal obvious difference (not shown). As with ADCB sensitivity, CFW sensitivity of the *rsp5-1* strain was also linked to formation of the K63-polyubiquitin conjugates. Overexpression of wild-type ubiquitin suppressed the CFW sensitivity of the *rsp5-1* mutants, while overexpression of K63R ubiquitin did not, and in fact, led to the increased CFW sensitivity (Figure 3.7B). Consistent with this notion, the K63R-only ubiquitin strain (K63R ubiquitin as the sole source of ubiquitin), itself, was also hypersensitive to CFW compared to the equivalent wild-type ubiquitin strain (Figure 3.7C), further indicating that formation of K63-polyubiquitin conjugates by Rsp5 is required for cell wall biogenesis and/or integrity.

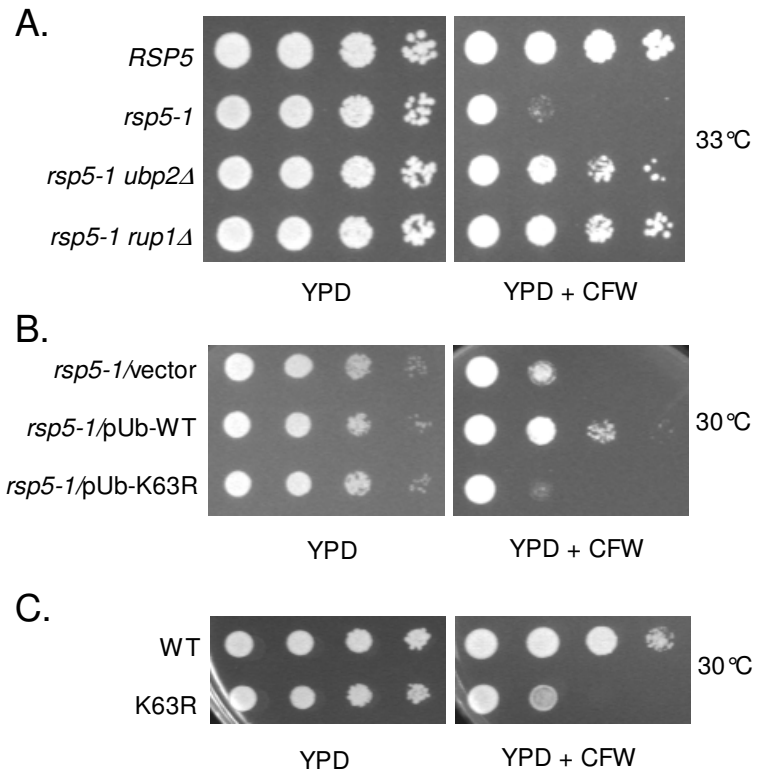


Figure 3.7 Ubp2 and Rup1 modulate effects of Rsp5 on cell wall homeostasis. A. Deletion of *ubp2* or *rup1* rescues CFW sensitivity of the *rsp5-1* strain. *RSP5* (FY56), *rsp5-1* (FW1808), *rsp5-1*/*ubp2*Δ (YK003), and *rsp5-1*/*rup1*Δ (YK004) cells were 6-fold serially diluted and plated on either YPD or YPD plates containing 5ug/ml of CFW. Cells were grown at 33°C for 4 days. B. Overexpression of wild type ubiquitin, but not K63R ubiquitin, rescues CFW sensitivity of *rsp5-1* strain. *rsp5-1* (FW1808) strain transformed with a empty plasmid, plasmid expressing wild type ubiquitin (pUb-WT; pUB39) or K63R ubiquitin (pUb-K63R; pUB197) were 10-fold serially diluted and

plated on YPD plates with or without 5ug/ml of CFW and grown at 30°C for 2 days. C. K63-linked polyubiquitination is required for resistance to CFW. Wild type ubiquitin (SUB492) and K63R-only ubiquitin cells (SUB493) were 10-fold serially diluted and plated on either YPD or YPD containing 7ug/ml of CFW and grown at 30°C for 3 days.

3.4 Discussion

Rsp5 is the first HECT E3 to be shown to display a distinct preference for assembly of K63-linked polyubiquitin chains *in vitro*. Human E6AP preferentially catalyzes K48 linkages, consistent with its role in targeting p53 for proteasomal degradation (195), while the synthesis of K63 chains by Rsp5 is consistent with reports that have shown that some Rsp5-mediated functions are dependent on K63 of ubiquitin (64, 66, 206). Rsp5 has also been linked to proteasomal degradation pathways (10, 57), which are predicted to involve K48 or K29 linkages (175). The type of chain synthesized by Rsp5 might be regulated by additional factors or perhaps substrate dependent. It should be noted that the preferential assembly of K63 chains by Rsp5 was seen when the activating E2 enzyme was either yeast Ubc1, human UbcH7, or Arabidopsis Ubc8 (not shown), suggesting that K63 chain assembly is an inherent characteristic of Rsp5 and not a function of the activating E2 enzyme. While Rsp5 is the only HECT E3 shown to preferentially catalyze K63 polyubiquitin linkages, the determinants that confer this specificity, relative to K48-specific HECT E3s (*e.g.*, human E6AP), remain to be identified. The strong similarity of Rsp5 to the Nedd4 family of mammalian HECT E3 suggests that these enzymes might also preferentially catalyze K63-linked polyubiquitination.

The chain specificity of Ubp2 was the same in the absence or presence of Rup1, indicating that this is an inherent characteristic of the enzyme, rather than an Rsp5- or Rup1-dependent effect. A few DUBs have been reported to disassemble both K48- and K63-linked chains in vitro, including Cezanne/A20 (59, 61, 232) Ubp14/isopeptidase T

(61), and CYLD (127, 220), while AMSH, a JAMM motif isopeptidase, has been shown to have a strong preference for disassembly of K63 chains (157). Consistent with the *in vitro* activity, the data indicated that Ubp2 modulates Rsp5-dependent K63-linked polyubiquitination *in vivo.* To my knowledge, the only *S. cerevisiae* enzymes other than Rsp5 that are known to be dedicated to the generation of K63-linked polyubiquitin chains in *S. cerevsiae* are the Mms2/Ubc13 E2 enzyme complex, which functions in the Rad6-dependent DNA damage tolerance pathway (97). While other DUBs in yeast are likely to have activity against K63-linked chains, Ubp2 is the only yeast deubiquitinating enzyme that is known to have a strong preference for disassembly of K63-linked conjugates over K48-linked conjugates, both *in vitro* and *in vivo*. While the substrate selectivity of Mms2/Ubc13 appears to be very restricted in yeast, the dramatic effect of the *ubp2*Δ mutation on accumulation of total K63-linked conjugates, along with the fact that Rsp5 has a broad range of target proteins, suggests that Rsp5 and Ubp2 may modulate a significant fraction of total K63 conjugation activity in yeast.

A previous report showed that an *rsp5* mutant exhibited increased cell wall chitin levels and increased sensitivity to CFW (114), and the *rsp5-1* mutant was shown here to be hypersensitive to CFW. A reflection of a potential cell wall defect of the *rsp5-1* was our previous observation that the temperature sensitivity of the *rsp5-1* strain could be rescued by sorbitol, an osomotic stabilizer (120). The fact that both *ubp2* and *rup1* mutations rescued the CFW sensitivity of the *rsp5-1* mutant is consistent with the previous report that the Rup1/Ubp2 deubiquitinating complex antagonizes the function of Rsp5 (see Chapter 2). It was shown that an *rsp5* mutant displays a significant increase in

the chitin level of cell wall compared to wild type cells, suggesting that the basis for the sensitivity of the *rsp5* mutant to CFW is due to the elevated chitin level, which facilitates binding of the drug. Furthermore, electron micrography analysis revealed that the *rsp5* mutant displayed abnormally thick cell wall structure. It will be important to see whether deletion of Rup1 or Ubp2 rescues the aberrant cell wall phenotypes of *rsp5-1*. My preliminary studies on CFW staining of cell walls did not reveal difference among the mutants, however, the fact that the staining of wile type *RSP5* and *rsp5-1* cells also looked similar (not shown) suggests that CFW staining is not a sensitive assay for cell wall phenotypes.

K63-linkages are known to be involved in at least some Rsp5-mediated processes, including endocytosis of plasma-membrane proteins such as Gap1 and Fur4 (66, 206) and regulation of mitochondrial distribution (64). There is no direct evidence that K63-linkages are involved in activation of Spt23 or the RNA export function of Rsp5, and we cannot rule out the possibility that other types of chains are catalyzed by Rsp5 in these pathways. It was previously proposed that Spt23 is monoubiquitinated by Rsp5 (180). While the vast majority of polyubiquitin chains that accumulated in the *ubp2*Δ cells were K63-linked, a small increase in total conjugates seen in the *ubp2*Δ*/K63R* suggested that Ubp2 may be capable of catalyzing deconjugation of other types of chains, or possibly removing monoubiquitin linkages from target proteins. With regard to the latter, Ubp2 can reverse the *in vitro* ubiquitination of certain Rsp5 substrates completely, regenerating the unmodified target protein (120) (see Chapter 2). However, the small increase of ubiquitin conjugates in *ubp2*Δ mutants might represent monoubiquitinated species,

raising an interesting possibility that Ubp2 might have cooperative function in promoting the monoubiquitination with Rsp5 for some substrates, where Rsp5 first catalyzes polyubiquitination of a substrate, with Ubp2 generating a monoubiquitinated form of the protein by disassembly of the chain but leaving the proximal ubiquitin in place (see Chapter 4 for further discussion). This might explain how Rsp5, which appears to be a highly processive enzyme *in vitro* on most of the substrates,, can generate monoubiquitinated proteins *in vivo*, such as monoubiquitinated Spt23, Rvs167, or Vps9 (180, 204, 209).

Data described in this chapter suggest that Rsp5 and Ubp2 balance at least some of the physiological processes in yeast, including cell wall biogenesis and ADCB sensitivity. The regulatory mechanism of Rsp5 and Ubp2 was shown to primarily involve K63-linked polyubiquitin chains. Illustration 3.2 describes the model for the balanced activity of Rsp5 and Ubp2. *ubp2*Δ cells seem to have no obvious phenotypes under physiologically normal condition, suggesting that hyperactivity of Rsp5 is not harmful for the normally growing cells. Alternatively, it is conceivable that there might be another DUB that plays a redundant role in terms of Rsp5 activity, although this is not likely the case because the only K63-specific DUB activity copurified with Rsp5 seems to be Ubp2 (see figure 5.1 in Chapter 5). The *ubp2*Δ mutant, however, displays hypersensitivity to ADCB, suggesting that the accumulation of K63-linked polyubiquitination of one or more substrates can be toxic under stress conditions. The ADCB sensitivities of *ubp2*Δ or *rup1*Δ have been suggested to be linked to the Gap1 general amino acid transporter activity, since the mutations led to the stabilization of the GFP-Gap1 in the

plasmamembrane under nitrogen-replete condition, where Gap1 is normally destabilized by endocytosis (182). However, the basis for the ADCB sensitivity in *ubp2*Δ mutants is complicated by the fact that K63R ubiquitin, which presumably inhibits Gap1 internalization (206), rescues the phenotype. Therefore, I hypothesize that accumulation of one or more of the K63-linked substrates, with unknown identities, confer sensitivity to the drug. Consistent with this notion, *ubp2*Δ mutant was shown to be sensitive to canavanine and cadmium (not shown).

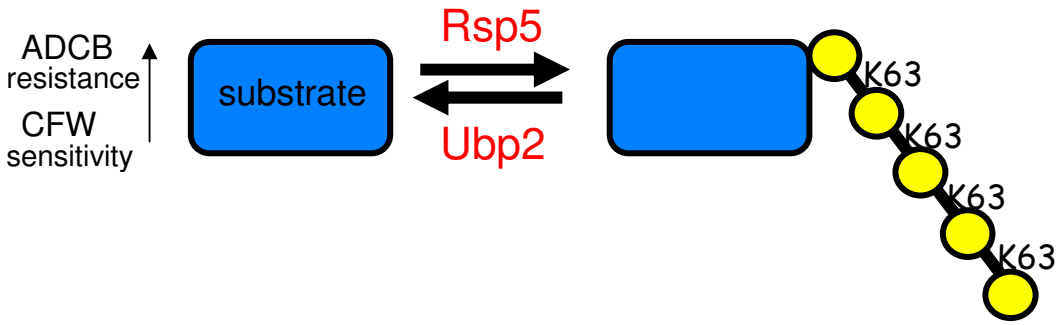


Illustration 3.2 Model for the regulation of K63-linked polyubiquitination by Rsp5 and Ubp2. Rsp5 and Ubp2 regulate K63-linked polyubiquitination of unknown substrates (see discussion in chapter 4). Accumulation of one or more of K63-linked polyubiquitinated substrates lead to hypersensitivity to ADCB, while defect in Rsp5 activity lead to sensitivity to CFW, potentially by altering chitin level.

Unlike the well-characterized role of K48-linkages in targeting proteins to the 26S proteasome, the biochemical function of the K63-linkages is unclear. The function is generally considered to be non-proteolytic, with the best-characterized examples being in the NFkB signaling pathway, DNA repair pathway, and ubiquitin-mediated endocytosis. It has been suggested that K63-linked conjugation of certain substrate can be recognized by the proteasome *in vitro* (187), however the fact that K63 conjugates accumulate in *ubp2*Δ cells suggest that at least the bulk of K63-conjugated Rsp5 substrates are not shunted to the proteasome. The structure of K63-linked polyubiquitin chains are distinct from K48 chains (Illustration 2.3) (221), consistent with distinct functional roles of the chain types. The identification of Rsp5 and Rup1/Ubp2 as a group of enzymes that specifically modulate K63 chain formation *in vivo* will allow further exploration of the precise biochemical function of this polymeric protein modifier.

CHAPTER FOUR

Proteomic analysis of Rsp5-associated ubiquitinated proteins

4.1 Introduction

Rsp5 and Ubp2 form a stable complex to regulate a set of K63-linked polyubiquitination in *S. cerevisae* (120, 121). Although it is not known whether a subset or all the of Rsp5 substrates are regulated by Ubp2 in the cells, it appears that deubiquitination of significant pool of the Ubp2 substrates are dependent on Rsp5 activity, based on the analysis of the total ubiquitin conjugates (see Chapter 3). Furthermore, Ubp2 was shown to affect at least some of the Rsp5-mediated processes, including endocytic pathways (182), OLE pathway, and cell wall biogenesis (Chapter 2 and 3). However, identities of the specific substrates that are subject to co-regulation by Rsp5 and Ubp2 are unknown, as are the substrates conjugated via K63-linkages in cells, in general. The fact that the bulk of K63-linked conjugates are detected in the *ubp2*Δ cells indicates that the *ubp2*Δ cells provide an excellent tool for enriching the ubiquitinated conjugates, particularly K63-linkages, for subsequent purification and identification by mass spectrometry. Furthermore, these conjugates appear to be stable in the cell extracts, indicating that the conjugates are highly resistant to promiscuous proteolytic activities present in the extracts. Therefore, I set out to purify the ubiquitinated conjugates to better understand the regulatory mechanisms of Rsp5 and Ubp2.

Previous attempts to purify Rsp5-associated proteins led to the identification of several proteins, such as Ubp2, Bul1, and Rup1 (107, 120), These proteins appear to be stably interacting co-factors of Rsp5, rather than substrates of the E3 ligase (see Chapter 2). This suggests that the previous methods are not suitable for purifying less stable and

transient substrate proteins. Therefore, an alternative purification scheme is necessary for isolating the ubiquitinated proteins.

Here, I describe an alternative approach for identifying the ubiquitinated substrates of Rsp5, using a two-step purification method. The mass spectrometry analysis revealed many potential targets of Rsp5, including Rpb1. Two previously uncharacterized proteins, Csr2 and Ecm21, were among the target proteins identified and shown to be ubiquitinated by Rsp5 via K63-linkages and deubiquitinated by Ubp2 *in vitro*. Together, these results suggest that this modified proteomic approach might be useful in identifying an array of target proteins of Rsp5 and Ubp2 complex and provide basis for characterizing the regulatory mechanisms of Rsp5 in synthesizing K63-linkages.

4.2 Materials and methods

Yeast strains, media, and plasmids

A list of yeast strains is shown in Table 1. *csr2*Δ and *ecm21*Δ mutations were introduced into FW1808 strain using the same method to generate YK028 and YK029, respectively, using the PCR-based recombination methods as described in Chapter 2. The *rup1*Δ mutation was introduced into YK009 strain using *HIS3* selection, generating YK030. pUB39 is a URA3-marked plasmid that expresses wild type ubiquitin under the *CUP1* promoter and pUB197 is identical to pUB39, except for the K63R mutation (208) (see materials and methods in Chapter 3). *CSR2* and *ECM21* ORFs were PCR amplified from genomic DNA and cloned into the pYES2 vector encoding an N-terminal HA-epitope for *in vitro* and *in vivo* expression. For the expression of N-terminally TAP-tagged ubiquitin, the *UBI4* ORF was subcloned into the pYES2-NTAP vector (120). The C-terminal codons of *UBI4* encoding GG altered to AA codons for expression of NTAP-Ub-$AA_{75\text{-}76}$. For the CFW containing media, CFW (Sigma) was dissolved in water and added to YPD media as final concentrations of 5 or 7ug/ml where indicated.

Purification of ubiquitinated proteins

For the large scale purification of ubiquitinated proteins, 6 liters of *ubp2*Δ cells containing the N-terminally TAP-tagged ubiquitin were grown and the expression of tagged ubiquitin was induced by switching the cells to galactose media over night. Cell lysate was prepared as described above. IgG sepharose (Amersham Biosciences) was added to the cleared lysate and rotated for 2 hours at 4°C. The beads were washed 2 times

with the NP40 buffer and the final wash was done with TEV buffer (0.1% NP40, 150mM NaCl, 50mM Tris pH 7.0, 1mM DTT), before adding TEV protease to the beads. The TEV cleavage reaction was done for 2 hours at room temperature on a rotator. The eluted ubiquitinated proteins were purified on GST-Rsp5C777A (GST-Rsp5-C-A) immobilized on glutathione sepharose by incubation for 2 hours at 4°C. The sepharose was washed 2 times with the 1% NP40 buffer followed by 0.1% NP40 buffer as a final wash, before they were analyzed on 10% SDS-PAGE gel and stained by Coomassie blue. For purification of endogenous ubiquitinated proteins, cell extracts were prepared using same method from 6L of *ubp2*Δ cells grown in YPD and bound to either GST-Rsp5-C-A or GST-Ubp2-C-S that are immobilized on glutathione sepahrose. Bands were excised from a Coomassie blue stained gel and subjected to in gel-tryptic digest. The fragmented peptides were analyzed by LC/MS (City of Hope mass spectrometry facility).

Protein interaction assays

GST-fusions of Rsp5 and Ubp2C-S were expressed from pGEX6p-1 in *Escherichia.coli* DH5α and purified on glutathione sepharose, as described previously (120). ^{35}S-labeled Csr2 and Ecm21 were produced using a coupled *in vitro* transcription/translation rabbit reticulocyte system (Promega) for *in vitro* GST-pulldown assays. The translated products were bound to GST-Rsp5 immobilized on glutathione sepharose for 2 hours at 4°C before they were analyzed on SDS-PAGE gel and autoradiography. HA-tagged Csr2 and Ecm21 were expressed under galactose induction in either wild type (FY56) or NTAP-RSP5 (YK001) strain. Approximately 30 O.D. of

cells were grown and resuspended with 1% NP40 lysis buffer (1% NP-40, 150mM NaCl, 10mM Tris 8.0) supplemented with protease inhibitors before the cells were broken up by bead beater. The extracts were cleared by centrifugating at 13000 rpm for 10 minutes and 20ul of IgG sepharose (Sigma) were added to the cleared cell extracts. After 2 hours of incubation at 4C, beads were collected by centrifugating at 25,000 rpm, washed with the same buffer 3 times, and resuspended with 1X SDS-loading buffer before analyzed by 8% SDS-PAGE. The gels were electroblotted onto nitrocellulose membrane for western blotting analysis. The presence of HA-Csr2 and Ecm21 was analyzed by anti-HA antibody (Santacruz biotech.) and NTAP-Rsp5 was analyzed by anti-TAP antibody (Rockland).

In vitro ubiquitination/deubiquitination assays

In vitro ubiquitination and deubiquitination assays were performed in the presence of 10mM Tris pH 7.5, 50mM NaCl, 5mM ATP, 5mM $MgCl_2$, 0.1mM DTT, and 50ug/ml ubiquitin (Sigma). Bacterially expressed Rsp5, Rup1, and Ubp2 were purified on glutathione sepharose and GST was removed by cleavage with PreScission protease (Amersham Biosciences). *In vitro* translated ^{35}S-labeled Csr2 or Ecm21, synthesized using transcription and translation coupled (TNT) rabbit reticulocyte lysate (Promega), were used as substrates. The ubiquitination reactions were performed as previously described (120). Ubiquitination reactions were carried out for 30 min at room temperature, followed by an additional 30 min for deubiquitination by Rup1/Ubp2. For the assays using K0, K48-only, K63-only ubiquitin (Boston Biochem), the *in vitro*

translated substrates were partially purified by DEAE anion exchange column to remove endogenous ubiquitin. The reactions were performed as describe above and stopped by addition of SDS-PAGE loading buffer and the samples were analyzed on 8% SDS-PAGE gel, followed by autoradiography. The deubiquitination assays using free ubiquitin chains utilized 3 mg of either K48 or K63 polyubiquitin chains 3-7 (Boston Biochem), and were incubated with 0.15–15 ng of Ubp2 for 1 h at room temperature in buffer containing 10mM Tris pH 7.5, 50mM NaCl, 5mM MgCl2, and 0.1mM DTT. The reactions were stopped by SDS–PAGE loading buffer and products were analyzed by 12% SDS–PAGE and staining with Coomassie blue.

Strain	Genotype	References
FY56	MATα *his4-912δR5 lys2-128Δ ura3-52*	(107)
FW1808	MATα *rsp5-1 his4-912δR5 lys2-128Δ ura3-52*	(107)
BY4741	MATa *his3 leu2 met15 ura3*	Open Biosystems
YK009	MATa *ubp2Δ*::KanMX6 *his3 leu2 met15 ura3*	Open Biosystems
YK028	MATα *rsp5-1 csr2Δ* ::KanMX6 *his4-912δR5 lys2-128Δ ura3-52*	(121)
YK029	MATα *rsp5-1 ecm21Δ*::KanMX6 *his4-912δR5 lys2-128Δ ura3-52*	(121)
YK030	MATa *ubp2Δ*::KanMX6 *rup1Δ*::HIS3 *leu2 met15 ura3*	(121)
YK032	MATa *ubp3Δ*::KanMX6 *his3 leu2 met15 ura3*	Open Biosystems
YK033	MATa *ubp4Δ* ::KanMX6 *his3 leu2 met15 ura3*	Open Biosystems

Table 4.1 List of the yeast strains used in chapter 4

4.3 Results

Physical association of K63-conjugated proteins with Rsp5 and Ubp2

Rsp5 has been shown to form direct and stable complexes *in vitro* with several of its substrates, generally mediated by the WW domains of Rsp5 and proline-containing motifs in the substrate proteins (227). The stable interactions between Rsp5 and its substrates seem to persist even after the substrates are ubiquitinated at least *in vitro*, as it was shown that the ubiquitinated forms of WBP2 still associate with GST-Rsp5 in pulldown assays (not shown). Because the K63 conjugates that accumulate in the *ubp2*Δ mutant are at least largely the result of Rsp5 ubiquitination activity, we predicted that conjugates in total *ubp2*Δ cell extract might stably interact with purified GST-Rsp5 *in vitro*. To test this, extracts were made from *UBP2* wild-type cells, *ubp2*Δ, *ubp3*Δ, and *ubp4*Δ mutant cells. While *ubp3*Δ and *ubp4*Δ mutant cells also accumulate ubiquitin conjugates to some degree (Figure 4.1), there is no known relationship between Rsp5 and either Ubp3 or Ubp4, and we therefore did not expect ubiquitinated proteins in these extracts to interact with Rsp5. As shown in Figure 4.1, GST-Rsp5 bound a large fraction of the input conjugates from the *ubp2*Δ cell extract, but not from any of the control extracts. This further substantiates that the conjugates that accumulate in the *ubp2*Δ mutants are likely to be a direct result of Rsp5-catalyzed ubiquitination.

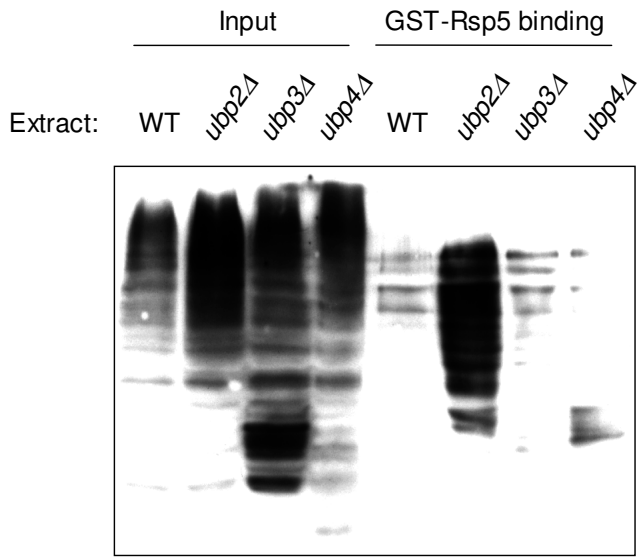


Figure 4.1 Binding of ubiquitin conjugates in *ubp2*Δ cells to GST-Rsp5.
Total cell extracts were prepared from wild type (BY4741), *ubp2*Δ (YK009), *ubp3*Δ (YK032), and *ubp4*Δ (YK033) strains and incubated with bacterially purified GST-Rsp5, immobilized on glutathione sepharose. Bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody.

Since the enrichment of the conjugates in *ubp2*Δ cells are due to the lack of Ubp2 activity (see Chapter 3), these conjugates are likely to be the direct substrates of Ubp2. Therefore, I predicted that the ubiquitin conjugates in *ubp2*Δ cells might be purified using

catalytically inactive GST-Ubp2 CS. As negative controls for the pulldown assay, Ubp6, a UBP member in yeast that associate with proteasome and another HECT E3, E6AP, was used. As expected, GST-Ubp2CS also bound to a significant fraction of conjugates in extract from *ubp2*Δ cells (Figure 4.2A and B), while GST-Ubp6 or another HECT E3, GST-E6AP, did not (Figure 4.2B). Because GST-Ubp2 can bind to Rup1 and Rsp5 present in cell extract (120), it was possible that the binding of GST-Ubp2 to conjugates was indirect and through Rup1 and Rsp5. This appears likely, since association of the conjugates with GST-Ubp2 was significantly decreased in the *ubp2*Δ*rup1*Δ double mutant (Figure 4.2A). However, a fraction of the conjugates were still bound in the absence of Rup1, suggesting that Ubp2 may be able to recognize some of its targets directly. This is consistent with the fact that Ubp2 can disassemble free K63-linked chains *in vitro* and can deubiquitinate Rsp5 substrates *in vitro* in the absence of Rup1, albeit with reduced efficiency (see Chapter 3).

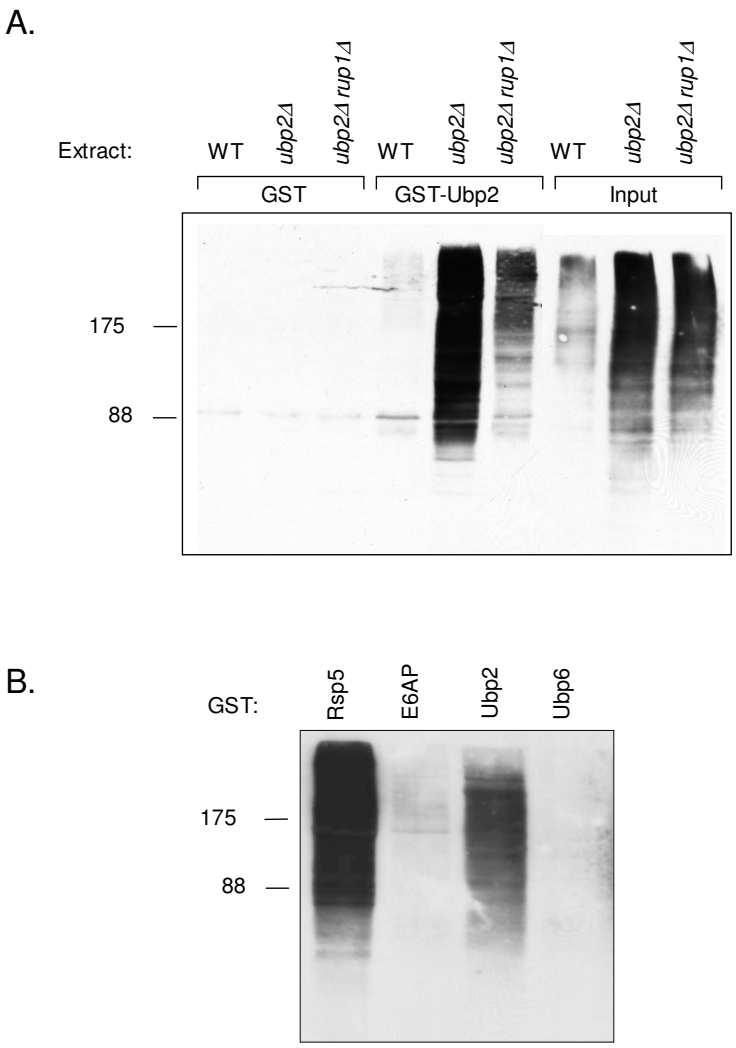


Figure 4.2 Binding of ubiquitin conjugates in *ubp2*Δ cells to GST-Ubp2-C-S. A. Cell extracts were prepared from wild type (BY4741), *ubp2*Δ (YK009), and *ubp2*Δ*rup1*Δ (YK030) strains and incubated with GST control or GST-Ubp2-C-S immobilized on glutathione sepharose. Bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin antibody. B. Cell extracts from *ubp2*Δ cells (YK009) were incubated with GST-Rsp5, GST-E6AP, GST-Ubp2-C-S, and GST-Ubp6 proteins immobilized on glutathione sepharose and analyzed as in A.

It is not known whether all targets of Rsp5, or only a subset of Rsp5 targets, are subject to potential regulation by Ubp2. The results shown in figure 4.1 suggested that we could identify the Ubp2-responsive substrates of Rsp5 by mass spectrometry-based identification of the proteins in the GST-Rsp5 pull-down from *ubp2*Δ cell extract. For this purpose, I expressed amino-terminally TAP-tagged ubiquitin in *ubp2*Δ cells (177). Since it was shown that GST-ubiquitin can be efficiently utilized for conjugation *in vitro* (250), I reasoned that the TAP-tag, which is about similar size as GST, would be used for substrate ubiquitination in yeast cells. Whether or not cells can utilize the TAP-tagged ubiquitin as sole source of ubiquitin is unknown. TAP-ubiquitin conjugates were isolated on IgG sepharose, and the conjugates were released from IgG sepharose by cleavage of the TAP tag with TEV protease. As shown in figure 4.3A, the TAP-tagged ubiquitin formed conjugates in *ubp2*Δ cells, and the conjugates could be efficiently purified on IgG sepharose. To exclude the possibility that TAP-ubiquitin was merely serving as an acceptor for endogenous ubiquitin, thereby forming free polyubiquitin conjugates, TAP-ubiquitin was expressed in which the terminal di-glycine residues were altered to alanine residues (TAP-Ub-AA_{75-76}). The expectation was that since the TAP-Ub-AA_{75-76} is incompetent for conjugation, IgG purification should not result in purification of ubiquitin conjugates. As expected, no significant accumulation or purification of ubiquitin conjugates was detected with the AA_{75-76} mutant, indicating that the wild-type TAP-ubiquitin was indeed being conjugated to target proteins.

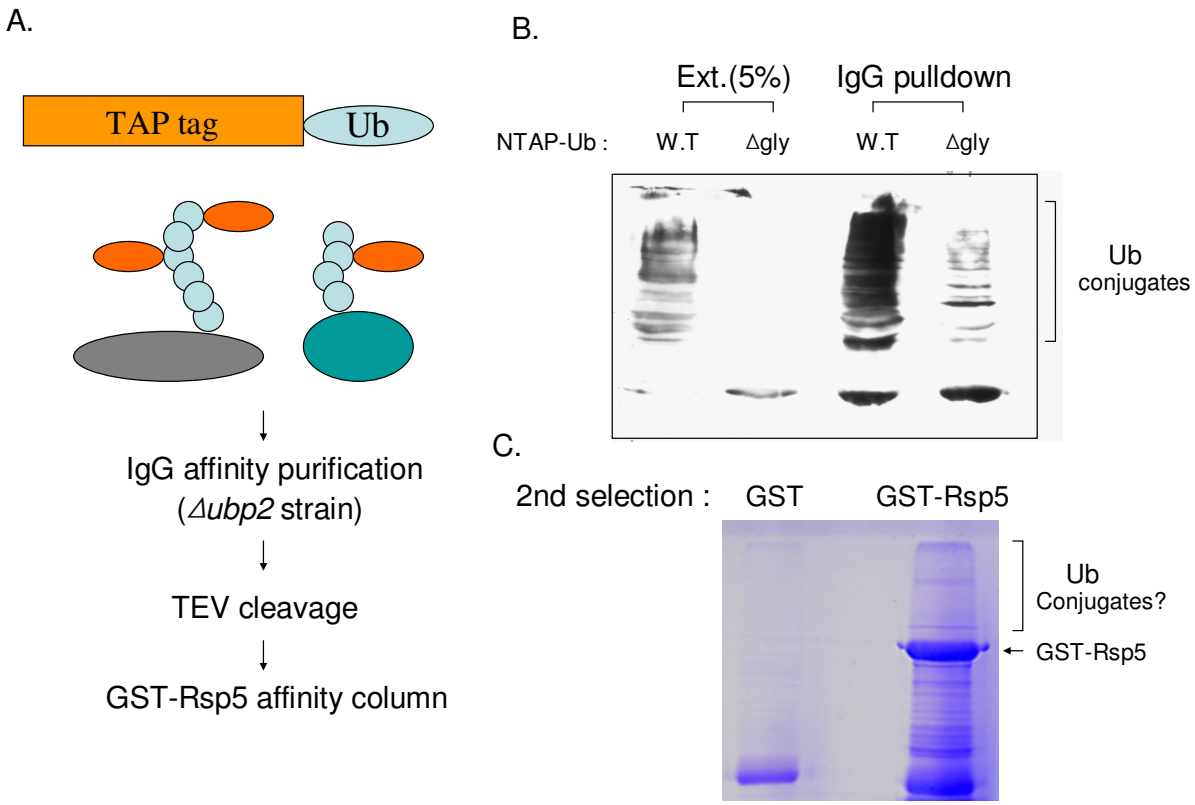


Figure 4.3 Purification of ubiquitin conjugates on GST-Rsp5. A. Purification scheme. B. N-terminally TAP-tagged wild type ubiquitin or ubiquitin $AA_{75\text{-}76}$ were expressed in *ubp2*Δ cells and conjugates purified on IgG sepharose. The samples were analyzed by SDS-PAGE and immunoblotting with anti-TAP antibody. C. Coomassie blue-stained gel of final eluates. 6 liters of the *ubp2*Δ cells expressing NTAP-Ub were subject to IgG sepharose, cleaved with TEV, and then purified on GST-Rsp5C-A immobilized on glutathione sepharose. The bound proteins were boiled in SDS-PAGE loading buffer and analyzed on 10% SDS-PAGE gel followed by Coomassie blue staining.

The purification was scaled up (starting with 6 liters of cells at $O.D._{600}$ of 1.5) for the two-step purification. First, IgG-sepharose was used for the first step purification to isolate substrates that are conjugated with TAP-ubiquitin. The conjugates were eluted with TEV protease and bacterially purified GST-Rsp5 immobilized on glutathione sepharose was used to purify Rsp5-specific ubiquitinated proteins. Figure 4.3C shows an example of a Coomassie blue-stained gel of the final eluate from a large-scale purification. As we were interested initially in the identity of the high molecular weight conjugates, the region of the gel above the migration point of GST-Rsp5-C-A was cut into several pieces and subject to mass spectrometry protein identification. As was the case in the NTAP-Rsp5 purification described in chapter 2, I speculated that most of the detectable protein bands lower than the size of GST-Rsp5 were degradation products of Rsp5. In separate large-scale experiments, GST-Rsp5-C-A as well as GST-Ubp2-C-S proteins immobilized on glutathione sepharose were also used to directly purify putatively ubiquitinated proteins from 6 liters of *ubp2*Δ cell extracts in one-step purification (Figure 4.2). Table 1 shows complete list of the proteins identified in multiple purifications which used GST-Rsp5-C-A proteins as baits, and Table 2 shows list of proteins identified in purifications used GST-Ubp2-C-S as bait. Rpb1, the largest subunit of RNA polymerase II, was identified again in this purification, strongly suggesting that it is a direct ubiquitination target of Rsp5 (107, 120) and shown to be the most abundant protein among the list of the proteins identified. Several proteins involved in RNA processing (Xrn1, Slh1, Rrp5, and Sen1) were among the proteins identified in the GST-Rsp5 purification, potentially suggesting that these might be unknown proteins

involved in the Rsp5-mediated RNA processing and/or nuclear export. Particularly, Sen1 contains multiple PxY or PY motifs, suggesting that it may directly bind to the WW domains of Rsp5. Rod1, a PxY motif-containing protein previously reported to bind to Rsp5 and implicated in drug resistance (7), was included in the list albeit in a small quantity, suggesting that the proteomic method isolates physiologically relevant target proteins. Notably, Pma1 and Ecm21 were identified in both GST-Rsp5 and GST-Ubp2 pulldown experiments, suggesting that these might be common substrates of Rsp5 and Ubp2. Interestingly, the activity of Pma1, the plasmamembrane H^+-ATPase, was genetically shown to be regulated by both Rsp5 and Ubp2 (40), further suggesting that it might be a direct target of both proteins. Rup1 was not isolated in the purification, suggesting that it is not significantly ubiquitinated by Rsp5, although it was shown to be ubiquitinated *in vitro* (not shown). Interestingly, Bul1, protein that binds to Rsp5 via its PPxY motif (243, 244), was among the list, suggesting that it might be ubiquitinated in the cells. It was also isolated in a previous proteomic studies that identified total ubiquitinated proteins from cells (171) and, in fact, it is efficiently ubiquitinated *in vitro* by Rsp5 in a PPxY motif-dependent manner (our unpublished result). It still remains unknown whether Rsp5-mediated ubiquitination of Bul1 and Bul2, two proteins that are known as cofactors of Rsp5 (206), has physiological relevance. Two proteins with unknown molecular function, Csr2 and Ecm21, were among the list and they were further analyzed in rest of the chapter.

Standard Name	SGD Name	Function
Rpb1	YDL140C	RNA polymerase II large subunit. Shown to be a substrate of Rsp5
Xrn1	YGL173C	5'-3' exonuclease component of cytoplasmic processing (P) bodies involved in mRNA decay. Also plays role in ribosomal RNA maturation
Csr2	YPR030W	Putatively involved in cell wall biogenesis
Ecm21	YBL101C	Putatively involved in chitin synthesis
Slh1	YGR271W	Putative RNA helicase related to Ski2p, involved in translation inhibition of non-poly(A) mRNAs
Tom1	YDR457W	E3 ubiquitin ligase of the Hect-domain class; has a role in mRNA export from the nucleus and may regulate transcriptional coactivators
Sen1	YJR430W	Nuclear protein, putative helicase required for processing of tRNAs, rRNAs, and small nuclear RNAs; potential Cdc28p substrate
Rrp5	YMR229C	Protein required for the synthesis of both 18S and 5.8S rRNA. Component of ribosomal subunit
Rpb2	YOR151C	RNA polymerase II second largest subunit B150
Spt5	YML010W	Protein that forms a complex with Spt4 and mediates both activation and inhibition of transcription elongation. Also plays role in pre-mRNA processing
Pma1	YGL008C	Plasma membrane H+-ATPase, pumps protons out of the cell
Bul1	YMR275C	Functional homolog of Bul2, overexpression causes missorting of amino acid permease. Shown to bind to Rsp5
Rpa1	YAR007C	Subunit of Replication Factor A (RF-A), a highly conserved single-stranded DNA binding protein involved in DNA replication, repair, and recombination
Rod1	YOR018W	Membrane protein; overexpression confers resistance to the GST substrate o-dinitrobenzene as well as to zinc and calcium. Shown to bind to Rsp5

Table 4.2 List of the proteins identified using GST-Rsp5-C-A

Standard Name	SGD Name	Function
Rsp5	YER125W	A multifunctional HECT-domain E3 ligase
Ecm21	YPR030W	Putatively involved in cell wall biogenesis
Pma1	YGL008C	Plasma membrane H+-ATPase, pumps protons out of the cell

Table 4.3 List of the proteins identified using GST-Ubp2-C-S.

Csr2 and Ecm21 are closely related proteins of approximately 125 kDa (Figure 4.4A), although both migrate as approximately 145 kDa on SDS-PAGE gels. Both have been shown previously to be ubiquitinated *in vivo* (156, 171, 216), although no connection to Rsp5 or Ubp2 was previously noted. Csr2 and Ecm21 are divergent over their first 180 amino acids, and are 32% identical over the remainder of their sequence. There are no identifiable motifs or functional domains and no biochemical function has been assigned to either protein, except for the fact that both proteins contain multiple PxY and PPxY motifs, suggesting that they might directly bind to WW domains of Rsp5. *CSR2* was previously isolated as a multicopy suppressor of a *chs5spa2* mutant, which exhibits aberrant cell wall structure and a defect in polarized cell growth (190). The *ecm21*Δ mutant was shown to be synthetically lethal with *chs1*Δ and *chs5*Δ, which are essential for chitin synthesis, suggesting a role for Ecm21 in cell wall integrity (219).

Since Rsp5 activity is previously implicated in cell wall biogenesis (121) (see Chapter 3) and the proteomics study suggests that Csr2 and Ecm21 are ubiquitinated proteins that bind to Rsp5, I decided to further investigate these two proteins in terms of regulation by Rsp5 and Ubp2.

First, direct binding activities of Csr2 and Ecm21 to Rsp5 were investigated. *In vitro* translated (rabbit reticulocyte lysate) ^{35}S-labelled Csr2 and Ecm21 was shown to bind efficiently to purified GST-Rsp5, while an unrelated protein (*in vitro* translated p53) did not (Figure 4.4B), suggesting that they bind directly to Rsp5. Preliminary domain mapping study did not reveal the determinants in Csr2 and Ecm21 required for binding to Rsp5, although it was shown that the C-terminal PPRY sequences in both proteins are dispensable for the interactions, suggesting that other upstream PxY motifs might be involved in the interactions. To test if Csr2 and Ecm21 are associated with Rsp5 in cells, HA-tagged Csr2 and Ecm21 were expressed in the *TAP-RSP5* (YK001) strain and pulldown experiments were performed. As expected, both HA-Csr2 and Ecm21 proteins were detected in the TAP-Rsp5 pulldown, suggesting that they associate with Rsp5 *in vivo* (Figure 4.4C).

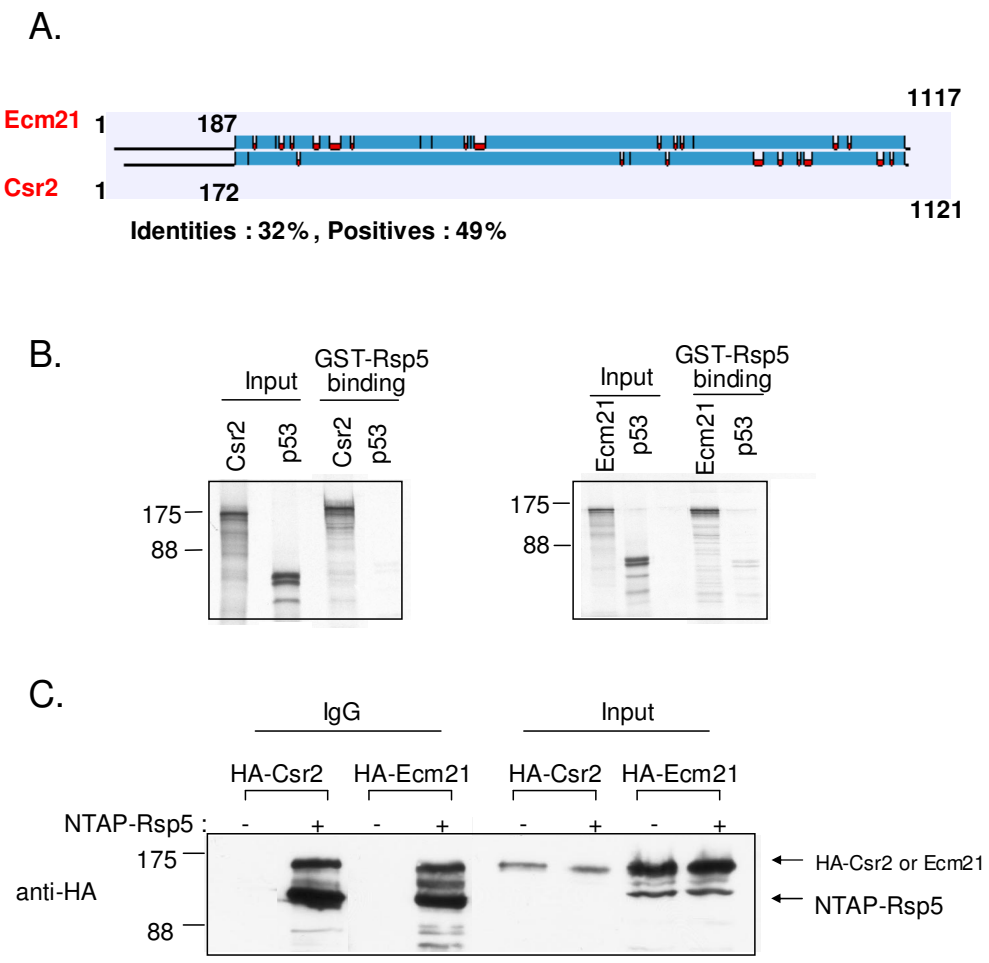


Figure 4.4 Csr2 and Ecm21 bind to Rsp5. A. Schematic diagram for overall sequence homology between Csr2 and Ecm21. B. *In vitro* translated ^{35}S-labeled Csr2 or Ecm21 were incubated with GST-Rsp5 immobilized on glutathione sepharose. Bound proteins were detected by SDS-PAGE and autoradiography. *In vitro* translated p53 was used as a negative control. C. HA-tagged Csr2 and Ecm21 were overexpressed in either wild type (FY56) strain or *NTAP-RSP5* (YK001) strain. Cell extracts were prepared and NTAP-Rsp5 was purified using IgG sepahrose and HA-proteins were detected by anti-HA western blotting.

Figure 4.5 shows that Rsp5 efficiently polyubiquitinated *in vitro* translated Csr2 and Ecm21. To test whether Csr2 and Ecm21 were polyubiquitinated via K63-linkages, the *in vitro* assay was performed in the presence of ubiquitin mutants (Figure 4.5B). The efficiency of polyubiquitination of Csr2 and Ecm21 was comparable when using wild type ubiquitin and K63-only ubiquitin, while the length of the chains was shorter with K48-only ubiquitin. The K48-only products were similar to those with K0 (no lysine) ubiquitin, suggesting that the majority of the products formed with K48-only ubiquitin are likely to represent multiple lysines being modified with single ubiquitin moieties. This is consistent with a previous report that multiple lysine residues of Ecm21 are ubiquitinated (171).

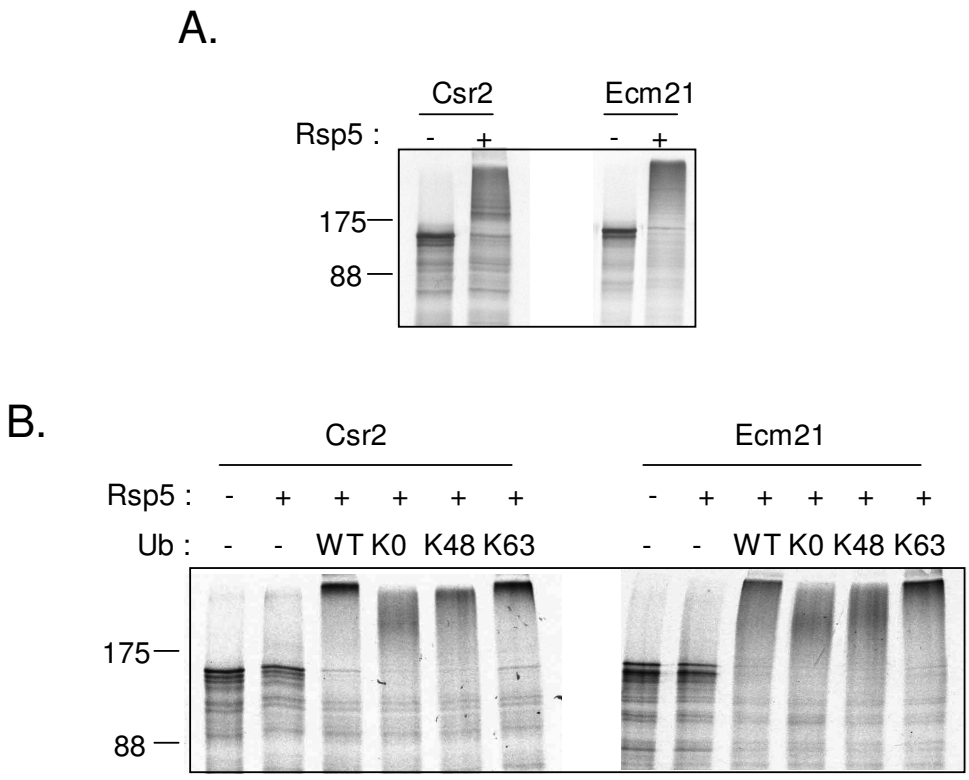


Figure 4.5 Csr2 and Ecm21 are substrates of Rsp5. A. *In vitro* translated ^{35}S-labeled Csr2 or Ecm21 were incubated with E1, E2, Ub, and ATP in the presence or absence of Rsp5 as described in materials and methods. Reactions were stopped after 30 min and the products were analyzed on SDS-PAGE and autoradiography. B. Rsp5-catalyzed ubiquitination reactions were carried out as in A, except that the endogenous ubiquitin present in the translated reactions were first removed by anion exchange chromatography. The reactions were then performed in the absence of added ubiquitin (lane 2) or the presence of wild type ubiquitin, K0 ubiquitin, K48-only ubiquitin, and K63-only ubiquitin (lanes 3-6). Products were analyzed as in B.

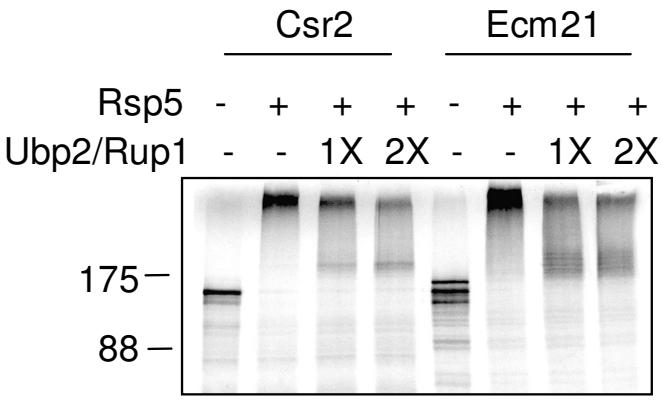


Figure 4.6 Ubp2 deubiquitinates Csr2 and Ecm21 *in vitro*. *In vitro* translated ^{35}S-labeled Csr2 or Ecm21 were incubated with E1, E2, Ub, and ATP in the presence or absence of Rsp5 as previously in figure 5. After 30 minutes of ubiquitination, Rup1 and Ubp2 proteins were added and additional 30 minutes were incubated. The final products were analyzed by 8% SDS-PAGE followed by autoradiography.

Figure 4.6 shows that the subsequent addition of Rup1 and Ubp2 to the Rsp5-catalyzed reaction led to deubiquitination of both Ecm21 and Csr2. Interestingly, we

did not observe recovery of the unmodified proteins even in the presence of excess amount of Ubp2, suggesting that Ubp2 may be unable to cleave the most proximal ubiquitin moieties from these substrates. This suggests a possible mechanism for generation of monoubiquitinated proteins by the combined actions of Rsp5 and Ubp2.

Since both *CSR2* and *ECM21* were previously implicated in cell wall biogenesis (190, 219) and Rsp5 has shown to be involved in cell wall biogenesis as well (121) (see Chapter 3), I investigated the potential genetic interactions between *CSR2/ECM21* and *RSP5* in the context of calcofluor white (CFW) sensitivity. While neither the *ecm21*Δ nor *csr2*Δ mutations lead to either enhanced or resistant sensitivity to CFW (not shown), both mutations partially suppressed the CFW sensitivity of the *rsp5-1* mutation (Figure 4.7). This suggests that deficient ubiquitination of either Csr2 or Ecm21 in the *rsp5-1* hypomorphic mutants might be at least the partial cause of the enhanced sensitivity of the strain to CFW, as eliminating either of the proteins resulted in partial rescue to the drug. This suggests a hypothetical model in which an abundance of unmodified Csr2 or Ecm21 leads to cell wall instability, while ubiquitination of Csr2/Ecm21, or lack of Csr2/Ecm21 entirely, leads to cell wall stabilization. The deletion of *csr2* or *ecm21* did not rescue the temperature sensitivity of the *rsp5-1* mutant, and multi-copy overexpression of either *CSR2* or *ECM21* changed neither CFW sensitivity nor temperature sensitivity of the *rsp5-1* strain (not shown).

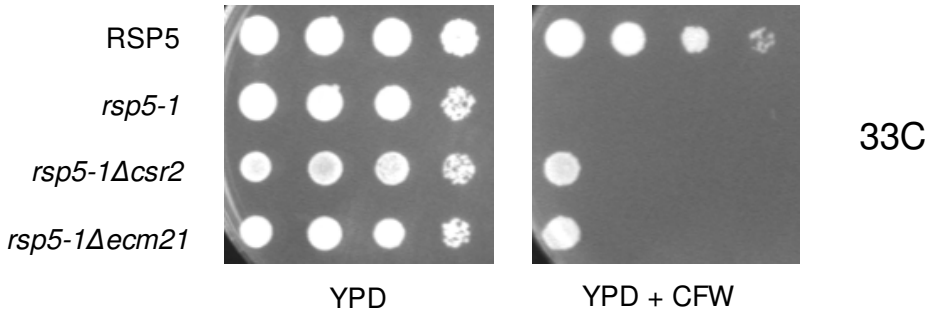


Figure 4.7 Deletions of either *csr2* or *ecm21* partially rescues *rsp5-1* phenotype. Wild type *RSP5* (FY56), *rsp5-1* (FW1808), *rsp5-1Δcsr2* (YK028), and *rsp5-1Δecm21* (YK029) cells were serially 6-fold diluted and spotted on either YPD or YPD containing 7ug/ml of CFW and grown at 33°C for 4 days.

4.4 Discussion

In this chapter, I described a proteomic approach for isolating ubiquitinated proteins that bind to Rsp5. Although it was not expected that the large TAP-tagging (~20kDa) of ubiquitin to be conjugated to all of the natural target proteins in cells, the TAP-ubiquitin have shown to be capable of being conjugated to at least some of the proteins (Figure 4.3B). Recently, there have been several reports that systematically isolated ubiquitinated conjugates from cells using various methods (156, 171, 216). All of these purifications were done under denaturing conditions using histidine-tagged ubiquitin, since the ubiquitination is sensitive to relatively abundant deubiquitinating enzymes present in the cell extracts. However, since the second step of my purification method requires naturally folded proteins that bind to GST-Rsp5, it was impossible to use denatured cell extracts. Furthermore, I did not expect that denaturing conditions were necessary for this purification project, since the increased K63-linked polyubiquitin chains in the *ubp2*Δ mutant cells appear highly stable in the cell extracts, unlike the K48-linked polyubiquitin chains that normally undergo rapid proteosomal-mediated degradation. This indirectly suggests that the K63-linked polyubiquitin chains might not be natural targets of proteasome in the cell extracts. Furthermore, specific DUB inhibitors such as NEM (N-ethylmaleimide) have been shown to efficiently block any promiscuous DUB activities in the cell extracts.

I isolated two closely related but largely uncharacterized proteins, Csr2 and Ecm21, as substrates of Rsp5, both of which appear related to cell wall integrity. *CSR2* was isolated as a multicopy suppressor of a *chs5*Δ*spa2*Δ mutant, which exhibits aberrant

cell wall structure and a defect in polarized cell growth (190). *ecm21*Δ mutation was shown to be synthetically lethal with *chs1*Δ and *chs5*Δ (219), both of which are essential for chitin synthesis, together suggesting that Csr2 and Ecm21 might be involved in a cell wall integrity pathway. Neither *ecm21*Δ nor *csr2*Δ mutations lead to either enhanced sensitivity or resistance to CFW. However, deletion of either *ecm21*Δ or *csr2*Δ partially rescued the CFW sensitivity of *rsp5-1*, suggesting possible roles of these proteins in the Rsp5-mediated cell wall homeostasis. It is possible that deletion of both *csr2* and *ecm2*1 simultaneously in the *rsp5-1* background might lead to further enhanced rescue of the mutant toward CFW, although the *csr2*Δ*ecm21*Δ double deletion mutant in *RSP5* wild type background was not shown to display either decreased or increased sensitivity to CFW (not shown).

Although both Csr2 and Ecm21 were previously shown to be ubiquitinated *in vivo* in large-scale ubiquitin proteomic projects (156, 171, 216), specific E3 enzymes responsible for their modification were not known. Csr2 and Ecm21 were both efficiently ubiquitinated via K63-linkages by Rsp5 *in vitro*, consistent with the fact that: 1) the CFW-sensitive phenotype of *rsp5-1* strain was rescued by overexpressing wild type ubiquitin, but not K63R ubiquitin, and 2) a strain expressing K63R-only ubiquitin as the sole source of ubiquitin was also sensitive to CFW (121) (Chapter 3). Therefore, we propose that Rsp5-catalyzed K63-ubiquitination of specific targets proteins, including but perhaps not limited to Csr2 and Ecm21, promotes cell wall biogenesis and/or stability. It remains to be determined whether Csr2 and Ecm21 are indeed modified via K63-linked

polyubiquitination by Rsp5 in the cells and what the precise biochemical functions of Csr2 and Ecm21 are and how these affect Rsp5-mediated cell wall homeostasis.

CHAPTER FIVE

Conclusion and future directions

In this work, I have shown that Rsp5 is physically coupled to the Ubp2 deubiquitinating enzyme and that its ability to catalyze K63-linked polyubiquitination is counterbalanced by the K63-linkage specific activity of Ubp2. This work provides opportunities to answer several important questions with regard to the regulation of HECT E3s and mechanisms of polyubiquitin chain synthesis.

Regulation of Rsp5/Ubp2 complex

The most important aspect of this work is that it represents a unique demonstration of a negative regulatory mechanism for a HECT E3 ligase. Our lab has long observed that Rsp5 efficiently produces polyubiquitination of its substrates *in vitro*, while several reports have shown that Rsp5 mediates mono- or short K63-linked polyubiquitination on several substrates *in vivo*, suggesting that there might be an unknown mechanism that is not reflected in the *in vitro* assays. My work suggests that, by being physically coupled to Ubp2, the inherently processive activity of Rsp5 might be counter-balanced to achieve regulated ubiquitination, or alternatively, to produce mono- or short oligo- K63-linkages. The former is consistent with our genetic studies (Chapter 2) in which the phenotype of overproduction of *UBP2* mimicked that of *rsp5* null mutant, whereas deletion of *ubp2* or *rup1* rescued *rsp5-1* temperature sensitivity. Several pieces of data supported the latter scenario, however, which showed that deletion of *ubp2* resulted in stabilization of Gap1 on the plasmamembrane (182), which phenocopies *rsp5* mutation. Furthermore, the *in vitro* assays using Csr2 and Ecm21 showed that Ubp2 activities do not generate complete reversion of the ubiquitinated substrates to

unmodified forms (Chapter 4), suggesting that Ubp2 might help promoting synthesis of mono- or oligo-K63-linkages. A current hypothesis is that Ubp2 might antagonize the majority of Rsp5-mediated K63-linked polyubiquitination, while it may promote Rsp5 activity by assisting with the generation of mono- or oligoubiquitinated substrates. More comprehensive analyses of the substrates that are regulated by Rsp5 and Ubp2 will give us better insight. Figure 2.16 in chapter 2 showed that the polyubiquitinating activity of Rsp5 can be limited by coupling to Ubp2 that is present in cell extracts. Also, the data suggests that the only K63-linkage specific DUB associated with Rsp5 is Ubp2. Rsp5 was not shown to associate with K48-linkage specific DUB (not shown).

Regulatory mechanisms of mammalian Rsp5 homologues

This work is the first demonstration of a physical coupling between a HECT E3 and a DUB. It will be important to determine whether other HECT E3s also utilize a similar mechanism. There are nine homologues of Rsp5 in human cells, including Nedd4, Smurf1/2, and Itch (Illustration 5.1). Based not only on their sequence similarities but also based on similar functions, such as mediating endocytosis of membrane proteins (110), it is possible that the mammalian homologues are also regulated by similar mechanisms by physically associating with DUBs. The simplest and initial test for their potential association with DUBs might be tests for abilities of the GST-HECT E3s for pull-down of DUB activities from cell extracts. Interestingly, when GST-Rsp5 was pre-incubated with HeLa cell extracts, a potential K63-linkage specific DUB was co-purified, whose activity was reflected in the WBP2 deubiquitination assay (not shown).

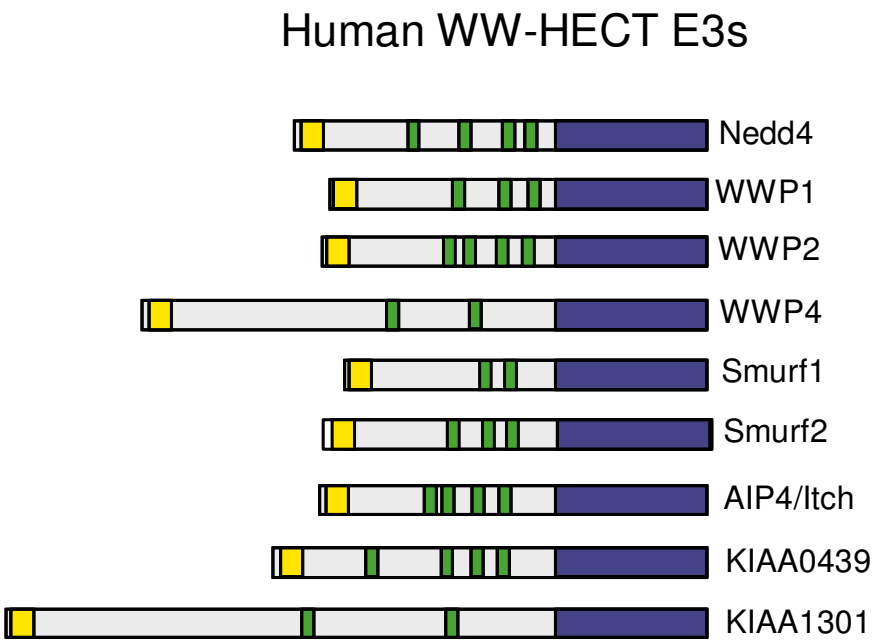


Illustration 5.1 A schematic diagram for nine human homologes of Rsp5. Domain colored with yellow, green, and blue indicate the C2 domain, WW domains, and HECT domain, respectively.

This might be due to the high structural similarities between Rsp5 and its mammalian homologues and Rsp5 is able to interact with a potential DUB that normally associate

with its homologues, similar to the fact that WW domains of five different Rsp5 homologues can interact with WBP2 (our unpublished result). An alternative approach to test for the potential DUB association is to use DUB-specific active-site probe ubiquitin-vinylsulfone (UbVs). UbVs is a C-terminally modified ubiquitin derivative which irreversibly modifies a subset of UCHs and UBPs members, and has successfully been shown to label several DUBs present in cell extracts, including Ubp2 (14, 15). If a HECT E3 is physically associated with a DUB, the immunopurified or GST-pulled-down of the HECT E3 protein complexes might be labeled with UbVS. It will be of interest to identify the potential DUBs associated with Nedd4, Smurf1/2, or Itch and test if they affect the activities of the E3s.

Determinants of chain type specificity

I have shown that Rsp5 preferentially catalyzes K63-linked polyubiquitination on WBP2, Ecm21, and Csr2 *in vitro*. This apparent *in vitro* preference to K63-linkages is consistent with previous reports that Fur4 and Gap1, two membrane proteins that are downregulated by Rsp5, are polyubiquitinated via K63-linkages (66, 206). These results strongly suggest that it is the inherent activity of Rsp5 that preferably assembles K63-linkages, not substrate-dependent activities. Likewise, E6AP assembled K48-linked polyubiquitination on two different substrates, p53 and Scribble, consistent with E6AP's demonstrated role in the degradation of the substrates (106, 165). It is important to note that the *in vitro* assays for Rsp5 and E6AP-mediated ubiquitination produced two different chain linkages when same E2, UbcH7, was used (see Chapter 3), excluding the

possibility that E2 might be the determinant of the differential chain type synthesis. Furthermore, Rsp5 preferably assembled K63-linked polyubiquitination on WBP2 when three different E2s (yeast Ubc1, human UbcH7, Arabidopsis Ubc8) were used (Chapter 3, not shown). Therefore, it is likely that the determinants for the preference of the different chain types (*e.g.*, K48 vs K63) lie on the E3 proteins. A next challenge is to map the determinants of the chain specificity within the E3s. One possible model is that the inherent conformation within the HECT domain might bring the thioster-conjugated ubiquitin to the proximity of the selective lysine (K63) on the preceding ubiquitin, which will allow the selective nucleophile attack from K63. Structural studies in combination with functional assays need to be done to test this hypothesis. The information from this study will give us insight into how Rsp5 promotes non-proteolytic K63-linked ubiquitination on its substrates, as opposed to proteolytic K48-linkages.

The study on the chain type specificities of HECT E3s can be extended to human homologues of Rsp5. It was shown that human Nedd4 can functionally substitue for the endocytic functions of Rsp5 (65), suggesting that Nedd4 might catalyze K63-linked polyubiquitination of membrane-associated substrates. However, the well established roles of Smuf1/2 and Itch include promoting the degradation of their substrates (149, 249, 251) (discussed in detail in Chapter 1), suggesting that they might catalyze K48-linked polyubiquitination. These observations present several interesting questions. Do Smurf1/2 and Itch promote K48-linked polyubiquitination while Nedd4 does K63? What is the basis for the distinct biochemical activities among the closely related homologues? Interestingly, a recent report demonstrated that Itch promotes K29-linked

polyubiquitination on its membrane-bound substrate, Deltex, leading to endocytosis. Does this mean Itch mediates assembly of two different chain types depending on the substrates? These outstanding questions need to be clarified using biochemical approaches to define the determinants for different chain type preferences.

K63-polyubiquitinated substrates and their function

An interesting piece of data described in chapter 3 is that a significant pool of K63-linked polyubiquitinated conjugates is enriched in *ubp2*Δ mutant cells. Considering our current lack of knowledge on the role of K63-linked polyubiquitination, *ubp2*Δ cells can be a useful tool for investigating the understudied ubiquitin chains types. The genetic studies in chapter 3 suggested that the accumulation of K63-linked substrates is responsible for sensitivity against the toxic drug ADCB, and for resistance to cell wall destabilizing drug calcofluor white, although specific substrates involved in those pathways still remains elusive. It will be interesting to compile a more comprehensive list of the specific substrates that are modified via K63-linkages in *ubp2*Δ and to determine physiological consequences of the modification on some of these substrates. For example, it is still unclear whether K63-polyubiquitin linked substrates are recognized and degraded by proteasome in cells. This issue might be able to explored using the K63-linked conjugates in *ubp2*Δ.

Budding yeast has proven to be an excellent model system to address many fundamental problems relevant to all eukaryotic organisms. Studies on Rsp5 and Ubp2 will lead to our enhanced understanding of not only the basis of regulatory mechanisms

for the mammalian HECT E3s, but also the mechanisms and functions of K63-linked polyubiquitination, which remains one of the most important challenges in the ubiquitin field. In the long run, the future work described above may assist designing effective strategies for regulating the activities of HECT E3s in medically important biological pathways.

Bibliography

1. **Aasland, R., T. J. Gibson, and A. F. Stewart.** 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. Trends Biochem Sci **20:**56-9.
2. **Ambroggio, X. I., D. C. Rees, and R. J. Deshaies.** 2004. JAMM: a metalloprotease-like zinc site in the proteasome and signalosome. PLoS Biol **2:**E2.
3. **Amerik, A., S. Swaminathan, B. A. Krantz, K. D. Wilkinson, and M. Hochstrasser.** 1997. In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. Embo J **16:**4826-38.
4. **Amerik, A. Y., and M. Hochstrasser.** 2004. Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta **1695:**189-207.
5. **Amerik, A. Y., S. J. Li, and M. Hochstrasser.** 2000. Analysis of the deubiquitinating enzymes of the yeast Saccharomyces cerevisiae. Biol Chem **381:**981-92.
6. **Amerik, A. Y., J. Nowak, S. Swaminathan, and M. Hochstrasser.** 2000. The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol Biol Cell **11:**3365-80.
7. **Andoh, T., Y. Hirata, and A. Kikuchi.** 2002. PY motifs of Rod1 are required for binding to Rsp5 and for drug resistance. FEBS Lett **525:**131-4.
8. **Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl, J. W. Harper, and S. J. Elledge.** 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86:**263-74.
9. **Balakirev, M. Y., S. O. Tcherniuk, M. Jaquinod, and J. Chroboczek.** 2003. Otubains: a new family of cysteine proteases in the ubiquitin pathway. EMBO Rep **4:**517-22.
10. **Beaudenon, S. L., M. R. Huacani, G. Wang, D. P. McDonnell, and J. M. Huibregtse.** 1999. Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large subunit of RNA polymerase II in Saccharomyces cerevisiae. Mol Cell Biol **19:**6972-9.
11. **Bienz, M.** 2006. The PHD finger, a nuclear protein-interaction domain. Trends Biochem Sci **31:**35-40.
12. **Bignell, G. R., W. Warren, S. Seal, M. Takahashi, E. Rapley, R. Barfoot, H. Green, C. Brown, P. J. Biggs, S. R. Lakhani, C. Jones, J. Hansen, E. Blair, B. Hofmann, R. Siebert, G. Turner, D. G. Evans, C. Schrander-Stumpel, F. A. Beemer, A. van Den Ouweland, D. Halley, B. Delpech, M. G. Cleveland, I. Leigh, J. Leisti, and S. Rasmussen.** 2000. Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet **25:**160-5.
13. **Bonni, S., H. R. Wang, C. G. Causing, P. Kavsak, S. L. Stroschein, K. Luo, and J. L. Wrana.** 2001. TGF-beta induces assembly of a Smad2-Smurf2

ubiquitin ligase complex that targets SnoN for degradation. Nat Cell Biol **3:**587-95.

14. **Borodovsky, A., B. M. Kessler, R. Casagrande, H. S. Overkleeft, K. D. Wilkinson, and H. L. Ploegh.** 2001. A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. Embo J **20:**5187-96.
15. **Borodovsky, A., H. Ovaa, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, H. L. Ploegh, and B. M. Kessler.** 2002. Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. Chem Biol **9:**1149-59.
16. **Brooks, C. L., and W. Gu.** 2006. p53 ubiquitination: Mdm2 and beyond. Mol Cell **21:**307-15.
17. **Brummelkamp, T. R., S. M. Nijman, A. M. Dirac, and R. Bernards.** 2003. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature **424:**797-801.
18. **Canning, M., C. Boutell, J. Parkinson, and R. D. Everett.** 2004. A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. J Biol Chem **279:**38160-8.
19. **Chastagner, P., A. Israel, and C. Brou.** 2006. Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep.
20. **Chastagner, P., A. Israel, and C. Brou.** 2006. Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep **7:**1147-53.
21. **Chau, V., J. W. Tobias, A. Bachmair, D. Marriott, D. J. Ecker, D. K. Gonda, and A. Varshavsky.** 1989. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science **243:**1576-83.
22. **Chen, D., C. L. Brooks, and W. Gu.** 2006. ARF-BP1 as a potential therapeutic target. Br J Cancer **94:**1555-8.
23. **Chen, D., N. Kon, M. Li, W. Zhang, J. Qin, and W. Gu.** 2005. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell **121:**1071-83.
24. **Chen, L., and K. Madura.** 2002. Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol Cell Biol **22:**4902-13.
25. **Chen, X., B. Zhang, and J. A. Fischer.** 2002. A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev **16:**289-94.
26. **Chen, Z. J.** 2005. Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol **7:**758-65.
27. **Chernova, T. A., K. D. Allen, L. M. Wesoloski, J. R. Shanks, Y. O. Chernoff, and K. D. Wilkinson.** 2003. Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. J Biol Chem **278:**52102-15.

28. **Chiu, R. K., J. Brun, C. Ramaekers, J. Theys, L. Weng, P. Lambin, D. A. Gray, and B. G. Wouters.** 2006. Lysine 63-polyubiquitination guards against translesion synthesis-induced mutations. PLoS Genet **2:**e116.
29. **Chow, M. K., J. P. Mackay, J. C. Whisstock, M. J. Scanlon, and S. P. Bottomley.** 2004. Structural and functional analysis of the Josephin domain of the polyglutamine protein ataxin-3. Biochem Biophys Res Commun **322:**387-94.
30. **Chung, C. H., and S. H. Baek.** 1999. Deubiquitinating enzymes: their diversity and emerging roles. Biochem Biophys Res Commun **266:**633-40.
31. **Ciechanover, A., S. Elias, H. Heller, and A. Hershko.** 1982. "Covalent affinity" purification of ubiquitin-activating enzyme. J Biol Chem **257:**2537-42.
32. **Ciechanover, A., H. Heller, S. Elias, A. L. Haas, and A. Hershko.** 1980. ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation. Proc Natl Acad Sci U S A **77:**1365-8.
33. **Ciehanover, A., Y. Hod, and A. Hershko.** 1978. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. Biochem Biophys Res Commun **81:**1100-5.
34. **Cooper, E. M., A. W. Hudson, J. Amos, J. Wagstaff, and P. M. Howley.** 2004. Biochemical analysis of Angelman syndrome-associated mutations in the E3 ubiquitin ligase E6-associated protein. J Biol Chem **279:**41208-17.
35. **Cope, G. A., G. S. Suh, L. Aravind, S. E. Schwarz, S. L. Zipursky, E. V. Koonin, and R. J. Deshaies.** 2002. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science **298:**608-11.
36. **Coscoy, L., and D. Ganem.** 2003. PHD domains and E3 ubiquitin ligases: viruses make the connection. Trends Cell Biol **13:**7-12.
37. **Cummins, J. M., and B. Vogelstein.** 2004. HAUSP is required for p53 destabilization. Cell Cycle **3:**689-92.
38. **D'Andrea, A. D.** 2003. The Fanconi road to cancer. Genes Dev **17:**1933-6.
39. **Dastur, A., S. Beaudenon, M. Kelley, R. M. Krug, and J. M. Huibregtse.** 2006. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. J Biol Chem **281:**4334-8.
40. **de la Fuente, N., A. M. Maldonado, and F. Portillo.** 1997. Glucose activation of the yeast plasma membrane H+-ATPase requires the ubiquitin-proteasome proteolytic pathway. FEBS Lett **411:**308-12.
41. **Debonneville, C., S. Y. Flores, E. Kamynina, P. J. Plant, C. Tauxe, M. A. Thomas, C. Munster, A. Chraibi, J. H. Pratt, J. D. Horisberger, D. Pearce, J. Loffing, and O. Staub.** 2001. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. Embo J **20:**7052-9.
42. **DeLorey, T. M., A. Handforth, S. G. Anagnostaras, G. E. Homanics, B. A. Minassian, A. Asatourian, M. S. Fanselow, A. Delgado-Escueta, G. D. Ellison, and R. W. Olsen.** 1998. Mice lacking the beta3 subunit of the GABAA receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. J Neurosci **18:**8505-14.
43. **Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen.** 2000. Activation of the IkappaB kinase complex by

TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103:**351-61.

44. **Deshaies, R. J.** 1999. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu Rev Cell Dev Biol **15:**435-67.
45. **Deveraux, Q., V. Ustrell, C. Pickart, and M. Rechsteiner.** 1994. A 26 S protease subunit that binds ubiquitin conjugates. J Biol Chem **269:**7059-61.
46. **Dodd, R. B., M. D. Allen, S. E. Brown, C. M. Sanderson, L. M. Duncan, P. J. Lehner, M. Bycroft, and R. J. Read.** 2004. Solution structure of the Kaposi's sarcoma-associated herpesvirus K3 N-terminal domain reveals a Novel E2-binding C4HC3-type RING domain. J Biol Chem **279:**53840-7.
47. **Doelling, J. H., N. Yan, J. Kurepa, J. Walker, and R. D. Vierstra.** 2001. The ubiquitin-specific protease UBP14 is essential for early embryo development in Arabidopsis thaliana. Plant J **27:**393-405.
48. **Duncan, K., J. G. Umen, and C. Guthrie.** 2000. A putative ubiquitin ligase required for efficient mRNA export differentially affects hnRNP transport. Curr Biol **10:**687-96.
49. **Dunn, R., and L. Hicke.** 2001. Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. Mol Biol Cell **12:**421-35.
50. **Dunn, R., and L. Hicke.** 2001. Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis. J Biol Chem **276:**25974-81.
51. **Dunn, R., D. A. Klos, A. S. Adler, and L. Hicke.** 2004. The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. J Cell Biol **165:**135-44.
52. **Dupre, S., and R. Haguenauer-Tsapis.** 2001. Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. Mol Cell Biol **21:**4482-94.
53. **Eddins, M. J., C. M. Carlile, K. M. Gomez, C. M. Pickart, and C. Wolberger.** 2006. Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat Struct Mol Biol **13:**915-20.
54. **Eisele, F., B. Braun, T. Pfirrmann, and D. H. Wolf.** 2006. Mutants of the deubiquitinating enzyme Ubp14 decipher pathway diversity of ubiquitin-proteasome linked protein degradation. Biochem Biophys Res Commun **350:**329-33.
55. **Eletr, Z. M., D. T. Huang, D. M. Duda, B. A. Schulman, and B. Kuhlman.** 2005. E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. Nat Struct Mol Biol **12:**933-4.
56. **Elsasser, S., R. R. Gali, M. Schwickart, C. N. Larsen, D. S. Leggett, B. Muller, M. T. Feng, F. Tubing, G. A. Dittmar, and D. Finley.** 2002. Proteasome subunit Rpn1 binds ubiquitin-like protein domains. Nat Cell Biol **4:**725-30.
57. **Erdeniz, N., and R. Rothstein.** 2000. Rsp5, a ubiquitin-protein ligase, is involved in degradation of the single-stranded-DNA binding protein rfa1 in Saccharomyces cerevisiae. Mol Cell Biol **20:**224-32.

58. **Etlinger, J. D., and A. L. Goldberg.** 1977. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc Natl Acad Sci U S A **74:**54-8.
59. **Evans, P. C., H. Ovaa, M. Hamon, P. J. Kilshaw, S. Hamm, S. Bauer, H. L. Ploegh, and T. S. Smith.** 2004. Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity. Biochem J **378:**727-34.
60. **Evans, P. C., T. S. Smith, M. J. Lai, M. G. Williams, D. F. Burke, K. Heyninck, M. M. Kreike, R. Beyaert, T. L. Blundell, and P. J. Kilshaw.** 2003. A novel type of deubiquitinating enzyme. J Biol Chem **278:**23180-6.
61. **Falquet, L., N. Paquet, S. Frutiger, G. J. Hughes, K. Hoang-Van, and J. C. Jaton.** 1995. A human de-ubiquitinating enzyme with both isopeptidase and peptidase activities in vitro. FEBS Lett **359:**73-7.
62. **Finley, D., and V. Chau.** 1991. Ubiquitination. Annu Rev Cell Biol **7:**25-69.
63. **Finley, D., S. Sadis, B. P. Monia, P. Boucher, D. J. Ecker, S. T. Crooke, and V. Chau.** 1994. Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Mol Cell Biol **14:**5501-9.
64. **Fisk, H. A., and M. P. Yaffe.** 1999. A role for ubiquitination in mitochondrial inheritance in Saccharomyces cerevisiae. J Cell Biol **145:**1199-208.
65. **Gajewska, B., N. Shcherbik, D. Oficjalska, D. S. Haines, and T. Zoladek.** 2003. Functional analysis of the human orthologue of the RSP5-encoded ubiquitin protein ligase, hNedd4, in yeast. Curr Genet **43:**1-10.
66. **Galan, J. M., and R. Haguenauer-Tsapis.** 1997. Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. Embo J **16:**5847-54.
67. **Gallagher, E., M. Gao, Y. C. Liu, and M. Karin.** 2006. Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. Proc Natl Acad Sci U S A **103:**1717-22.
68. **Gao, M., T. Labuda, Y. Xia, E. Gallagher, D. Fang, Y. C. Liu, and M. Karin.** 2004. Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. Science **306:**271-5.
69. **Gao, Q., A. Kumar, L. Singh, J. M. Huibregtse, S. Beaudenon, S. Srinivasan, D. E. Wazer, H. Band, and V. Band.** 2002. Human papillomavirus E6-induced degradation of E6TP1 is mediated by E6AP ubiquitin ligase. Cancer Res **62:**3315-21.
70. **Garrus, J. E., U. K. von Schwedler, O. W. Pornillos, S. G. Morham, K. H. Zavitz, H. E. Wang, D. A. Wettstein, K. M. Stray, M. Cote, R. L. Rich, D. G. Myszka, and W. I. Sundquist.** 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell **107:**55-65.
71. **Gewin, L., H. Myers, T. Kiyono, and D. A. Galloway.** 2004. Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. Genes Dev **18:**2269-82.
72. **Gitan, R. S., and D. J. Eide.** 2000. Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter. Biochem J **346 Pt 2:**329-36.

73. **Goldstein, G., M. Scheid, U. Hammerling, D. H. Schlesinger, H. D. Niall, and E. A. Boyse.** 1975. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. Proc Natl Acad Sci U S A **72:**11-5.
74. **Gong, B., Z. Cao, P. Zheng, O. V. Vitolo, S. Liu, A. Staniszewski, D. Moolman, H. Zhang, M. Shelanski, and O. Arancio.** 2006. Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory. Cell **126:**775-88.
75. **Goto, E., S. Ishido, Y. Sato, S. Ohgimoto, K. Ohgimoto, M. Nagano-Fujii, and H. Hotta.** 2003. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. J Biol Chem **278:**14657-68.
76. **Grant, P. A., L. Duggan, J. Cote, S. M. Roberts, J. E. Brownell, R. Candau, R. Ohba, T. Owen-Hughes, C. D. Allis, F. Winston, S. L. Berger, and J. L. Workman.** 1997. Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev **11:**1640-50.
77. **Gross-Mesilaty, S., E. Reinstein, B. Bercovich, K. E. Tobias, A. L. Schwartz, C. Kahana, and A. Ciechanover.** 1998. Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway. Proc Natl Acad Sci U S A **95:**8058-63.
78. **Grossman, S. R., M. E. Deato, C. Brignone, H. M. Chan, A. L. Kung, H. Tagami, Y. Nakatani, and D. M. Livingston.** 2003. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science **300:**342-4.
79. **Gurtan, A. M., P. Stuckert, and A. D. D'Andrea.** 2006. The WD40 repeats of FANCL are required for Fanconi anemia core complex assembly. J Biol Chem **281:**10896-905.
80. **Gwizdek, C., M. Hobeika, B. Kus, B. Ossareh-Nazari, C. Dargemont, and M. S. Rodriguez.** 2005. The mRNA nuclear export factor Hpr1 is regulated by Rsp5-mediated ubiquitylation. J Biol Chem **280:**13401-5.
81. **Harty, R. N., M. E. Brown, G. Wang, J. Huibregtse, and F. P. Hayes.** 2000. A PPxY motif within the VP40 protein of Ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. Proc Natl Acad Sci U S A **97:**13871-6.
82. **Hatakeyama, S., and K. I. Nakayama.** 2003. U-box proteins as a new family of ubiquitin ligases. Biochem Biophys Res Commun **302:**635-45.
83. **Hatakeyama, S., M. Yada, M. Matsumoto, N. Ishida, and K. I. Nakayama.** 2001. U box proteins as a new family of ubiquitin-protein ligases. J Biol Chem **276:**33111-20.
84. **Hein, C., J. Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. Andre.** 1995. NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol Microbiol **18:**77-87.

85. **Hellerbrand, C., E. Bumes, F. Bataille, W. Dietmaier, R. Massoumi, and A. K. Bosserhoff.** 2006. Reduced expression of CYLD in human colon and hepatocellular carcinomas. Carcinogenesis.
86. **Helliwell, S. B., S. Losko, and C. A. Kaiser.** 2001. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. J Cell Biol **153:**649-62.
87. **Hershko, A., H. Heller, S. Elias, and A. Ciechanover.** 1983. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem **258:**8206-14.
88. **Hershko, A., and I. A. Rose.** 1987. Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. Proc Natl Acad Sci U S A **84:**1829-33.
89. **Hetfeld, B. K., A. Helfrich, B. Kapelari, H. Scheel, K. Hofmann, A. Guterman, M. Glickman, R. Schade, P. M. Kloetzel, and W. Dubiel.** 2005. The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1. Curr Biol **15:**1217-21.
90. **Hettema, E. H., J. Valdez-Taubas, and H. R. Pelham.** 2004. Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. Embo J **23:**1279-88.
91. **Hicke, L.** 2001. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol **2:**195-201.
92. **Hicke, L., H. L. Schubert, and C. P. Hill.** 2005. Ubiquitin-binding domains. Nat Rev Mol Cell Biol **6:**610-21.
93. **Hirabayashi, M., K. Inoue, K. Tanaka, K. Nakadate, Y. Ohsawa, Y. Kamei, A. H. Popiel, A. Sinohara, A. Iwamatsu, Y. Kimura, Y. Uchiyama, S. Hori, and A. Kakizuka.** 2001. VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. Cell Death Differ **8:**977-84.
94. **Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis, and S. Jentsch.** 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature **419:**135-41.
95. **Hofmann, K., and P. Bucher.** 1996. The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway. Trends Biochem Sci **21:**172-3.
96. **Hofmann, K., and L. Falquet.** 2001. A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. Trends Biochem Sci **26:**347-50.
97. **Hofmann, R. M., and C. M. Pickart.** 1999. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell **96:**645-53.
98. **Hoppe, T.** 2005. Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. Trends Biochem Sci **30:**183-7.
99. **Hoppe, T., K. Matuschewski, M. Rape, S. Schlenker, H. D. Ulrich, and S. Jentsch.** 2000. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell **102:**577-86.

100. **Howley, P. M., K. Munger, H. Romanczuk, M. Scheffner, and J. M. Huibregtse.** 1991. Cellular targets of the oncoproteins encoded by the cancer associated human papillomaviruses. Princess Takamatsu Symp **22:**239-48.
101. **Hu, M., P. Li, M. Li, W. Li, T. Yao, J. W. Wu, W. Gu, R. E. Cohen, and Y. Shi.** 2002. Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde. Cell **111:**1041-54.
102. **Huang, L., E. Kinnucan, G. Wang, S. Beaudenon, P. M. Howley, J. M. Huibregtse, and N. P. Pavletich.** 1999. Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. Science **286:**1321-6.
103. **Huang, T. T., S. M. Nijman, K. D. Mirchandani, P. J. Galardy, M. A. Cohn, W. Haas, S. P. Gygi, H. L. Ploegh, R. Bernards, and A. D. D'Andrea.** 2006. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nat Cell Biol **8:**339-47.
104. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A **92:**5249.
105. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc Natl Acad Sci U S A **92:**2563-7.
106. **Huibregtse, J. M., M. Scheffner, and P. M. Howley.** 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. Embo J **10:**4129-35.
107. **Huibregtse, J. M., J. C. Yang, and S. L. Beaudenon.** 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. Proc Natl Acad Sci U S A **94:**3656-61.
108. **Ikeda, F., and I. Dikic.** 2006. CYLD in ubiquitin signaling and tumor pathogenesis. Cell **125:**643-5.
109. **Imai, Y., M. Soda, S. Hatakeyama, T. Akagi, T. Hashikawa, K. I. Nakayama, and R. Takahashi.** 2002. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell **10:**55-67.
110. **Ingham, R. J., G. Gish, and T. Pawson.** 2004. The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene **23:**1972-84.
111. **Johnson, E. S., P. C. Ma, I. M. Ota, and A. Varshavsky.** 1995. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J Biol Chem **270:**17442-56.
112. **Johnston, S. C., S. M. Riddle, R. E. Cohen, and C. P. Hill.** 1999. Structural basis for the specificity of ubiquitin C-terminal hydrolases. Embo J **18:**3877-87.
113. **Kaida, D., A. Toh-e, and Y. Kikuchi.** 2003. Rsp5-Bul1/2 complex is necessary for the HSE-mediated gene expression in budding yeast. Biochem Biophys Res Commun **306:**1037-41.

114. **Kaminska, J., M. Kwapisz, K. Grabinska, J. Orlowski, M. Boguta, G. Palamarczyk, and T. Zoladek.** 2005. Rsp5 ubiquitin ligase affects isoprenoid pathway and cell wall organization in S. cerevisiae. Acta Biochim Pol **52:**207-20.
115. **Kamura, T., S. Sato, D. Haque, L. Liu, W. G. Kaelin, Jr., R. C. Conaway, and J. W. Conaway.** 1998. The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes Dev **12:**3872-81.
116. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol **18:**621-63.
117. **Katzmann, D. J., S. Sarkar, T. Chu, A. Audhya, and S. D. Emr.** 2004. Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. Mol Biol Cell **15:**468-80.
118. **Kavsak, P., R. K. Rasmussen, C. G. Causing, S. Bonni, H. Zhu, G. H. Thomsen, and J. L. Wrana.** 2000. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell **6:**1365-75.
119. **Kawakami, T., T. Chiba, T. Suzuki, K. Iwai, K. Yamanaka, N. Minato, H. Suzuki, N. Shimbara, Y. Hidaka, F. Osaka, M. Omata, and K. Tanaka.** 2001. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. Embo J **20:**4003-12.
120. **Kee, Y., N. Lyon, and J. M. Huibregtse.** 2005. The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. Embo J **24:**2414-24.
121. **Kee, Y., W. Munoz, N. Lyon, and J. M. Huibregtse.** 2006. The Ubp2 deubiquitinating enzyme modulates Rsp5-dependent K63-linked polyubiquitin conjugates in Saccharomyces cerevisiae. J Biol Chem.
122. **Kelley, M. L., K. E. Keiger, C. J. Lee, and J. M. Huibregtse.** 2005. The global transcriptional effects of the human papillomavirus E6 protein in cervical carcinoma cell lines are mediated by the E6AP ubiquitin ligase. J Virol **79:**3737-47.
123. **Kikonyogo, A., F. Bouamr, M. L. Vana, Y. Xiang, A. Aiyar, C. Carter, and J. Leis.** 2001. Proteins related to the Nedd4 family of ubiquitin protein ligases interact with the L domain of Rous sarcoma virus and are required for gag budding from cells. Proc Natl Acad Sci U S A **98:**11199-204.
124. **Kim, I., K. Mi, and H. Rao.** 2004. Multiple interactions of rad23 suggest a mechanism for ubiquitylated substrate delivery important in proteolysis. Mol Biol Cell **15:**3357-65.
125. **Koegl, M., T. Hoppe, S. Schlenker, H. D. Ulrich, T. U. Mayer, and S. Jentsch.** 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell **96:**635-44.
126. **Komuro, A., T. Imamura, M. Saitoh, Y. Yoshida, T. Yamori, K. Miyazono, and K. Miyazawa.** 2004. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene **23:**6914-23.

127. **Kovalenko, A., C. Chable-Bessia, G. Cantarella, A. Israel, D. Wallach, and G. Courtois.** 2003. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature **424:**801-5.
128. **Krampe, S., O. Stamm, C. P. Hollenberg, and E. Boles.** 1998. Catabolite inactivation of the high-affinity hexose transporters Hxt6 and Hxt7 of Saccharomyces cerevisiae occurs in the vacuole after internalization by endocytosis. FEBS Lett **441:**343-7.
129. **Krsmanovic, T., and R. Kolling.** 2004. The HECT E3 ubiquitin ligase Rsp5 is important for ubiquitin homeostasis in yeast. FEBS Lett **577:**215-9.
130. **Kuballa, P., K. Matentzoglu, and M. Scheffner.** 2006. The role of the ubiquitin ligase E6-AP in human papillomavirus E6-mediated degradation of PDZ domain containing proteins. J Biol Chem.
131. **Lallemand, F., S. R. Seo, N. Ferrand, M. Pessah, S. L'Hoste, G. Rawadi, S. Roman-Roman, J. Camonis, and A. Atfi.** 2005. AIP4 restricts transforming growth factor-beta signaling through a ubiquitination-independent mechanism. J Biol Chem **280:**27645-53.
132. **Lammer, D., N. Mathias, J. M. Laplaza, W. Jiang, Y. Liu, J. Callis, M. Goebl, and M. Estelle.** 1998. Modification of yeast Cdc53p by the ubiquitin-related protein rub1p affects function of the SCFCdc4 complex. Genes Dev **12:**914-26.
133. **Leggett, D. S., J. Hanna, A. Borodovsky, B. Crosas, M. Schmidt, R. T. Baker, T. Walz, H. Ploegh, and D. Finley.** 2002. Multiple associated proteins regulate proteasome structure and function. Mol Cell **10:**495-507.
134. **Li, H., S. Ilin, W. Wang, E. M. Duncan, J. Wysocka, C. D. Allis, and D. J. Patel.** 2006. Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature **442:**91-5.
135. **Li, M., L. S. Becnel, W. Li, W. E. Fisher, C. Chen, and Q. Yao.** 2005. Signal transduction in human pancreatic cancer: roles of transforming growth factor beta, somatostatin receptors, and other signal intermediates. Arch Immunol Ther Exp (Warsz) **53:**381-7.
136. **Li, M., C. L. Brooks, F. Wu-Baer, D. Chen, R. Baer, and W. Gu.** 2003. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science **302:**1972-5.
137. **Li, M., D. Chen, A. Shiloh, J. Luo, A. Y. Nikolaev, J. Qin, and W. Gu.** 2002. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature **416:**648-53.
138. **Li, Z., D. Wang, E. M. Messing, and G. Wu.** 2005. VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha. EMBO Rep **6:**373-8.
139. **Li, Z., D. Wang, X. Na, S. R. Schoen, E. M. Messing, and G. Wu.** 2002. Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. Biochem Biophys Res Commun **294:**700-9.
140. **Linares, L. K., A. Hengstermann, A. Ciechanover, S. Muller, and M. Scheffner.** 2003. HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proc Natl Acad Sci U S A **100:**12009-14.

141. **Lindsey, D. F., A. Amerik, W. J. Deery, J. D. Bishop, M. Hochstrasser, and R. H. Gomer.** 1998. A deubiquitinating enzyme that disassembles free polyubiquitin chains is required for development but not growth in Dictyostelium. J Biol Chem **273:**29178-87.
142. **Liu, Y., L. Fallon, H. A. Lashuel, Z. Liu, and P. T. Lansbury, Jr.** 2002. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell **111:**209-18.
143. **Liu, Y. C.** 2004. Ubiquitin ligases and the immune response. Annu Rev Immunol **22:**81-127.
144. **Longtine, M. S., A. McKenzie, 3rd, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippsen, and J. R. Pringle.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953-61.
145. **Lorenzo, M. E., J. U. Jung, and H. L. Ploegh.** 2002. Kaposi's sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class major histocompatibility complexes to late endocytic compartments. J Virol **76:**5522-31.
146. **Lovering, R., I. M. Hanson, K. L. Borden, S. Martin, N. J. O'Reilly, G. I. Evan, D. Rahman, D. J. Pappin, J. Trowsdale, and P. S. Freemont.** 1993. Identification and preliminary characterization of a protein motif related to the zinc finger. Proc Natl Acad Sci U S A **90:**2112-6.
147. **Lu, Z., S. Xu, C. Joazeiro, M. H. Cobb, and T. Hunter.** 2002. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. Mol Cell **9:**945-56.
148. **Lyapina, S. A., C. C. Correll, E. T. Kipreos, and R. J. Deshaies.** 1998. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. Proc Natl Acad Sci U S A **95:**7451-6.
149. **Magnifico, A., S. Ettenberg, C. Yang, J. Mariano, S. Tiwari, S. Fang, S. Lipkowitz, and A. M. Weissman.** 2003. WW domain HECT E3s target Cbl RING finger E3s for proteasomal degradation. J Biol Chem **278:**43169-77.
150. **Makarova, K. S., L. Aravind, and E. V. Koonin.** 2000. A novel superfamily of predicted cysteine proteases from eukaryotes, viruses and Chlamydia pneumoniae. Trends Biochem Sci **25:**50-2.
151. **Mao, Y., F. Senic-Matuglia, P. P. Di Fiore, S. Polo, M. E. Hodsdon, and P. De Camilli.** 2005. Deubiquitinating function of ataxin-3: insights from the solution structure of the Josephin domain. Proc Natl Acad Sci U S A **102:**12700-5.
152. **Martin, G. S.** 2001. The hunting of the Src. Nat Rev Mol Cell Biol **2:**467-75.
153. **Massoumi, R., K. Chmielarska, K. Hennecke, A. Pfeifer, and R. Fassler.** 2006. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell **125:**665-77.
154. **Matsumoto, M., M. Yada, S. Hatakeyama, H. Ishimoto, T. Tanimura, S. Tsuji, A. Kakizuka, M. Kitagawa, and K. I. Nakayama.** 2004. Molecular clearance of ataxin-3 is regulated by a mammalian E4. Embo J **23:**659-69.

155. **Matsuura, T., J. S. Sutcliffe, P. Fang, R. J. Galjaard, Y. H. Jiang, C. S. Benton, J. M. Rommens, and A. L. Beaudet.** 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. Nat Genet **15:**74-7.
156. **Mayor, T., J. R. Lipford, J. Graumann, G. T. Smith, and R. J. Deshaies.** 2005. Analysis of polyubiquitin conjugates reveals that the Rpn10 substrate receptor contributes to the turnover of multiple proteasome targets. Mol Cell Proteomics **4:**741-51.
157. **McCullough, J., M. J. Clague, and S. Urbe.** 2004. AMSH is an endosome-associated ubiquitin isopeptidase. J Cell Biol **166:**487-92.
158. **Medicherla, B., Z. Kostova, A. Schaefer, and D. H. Wolf.** 2004. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. EMBO Rep **5:**692-7.
159. **Meulmeester, E., Y. Pereg, Y. Shiloh, and A. G. Jochemsen.** 2005. ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. Cell Cycle **4:**1166-70.
160. **Meyer, H. H., Y. Wang, and G. Warren.** 2002. Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. Embo J **21:**5645-52.
161. **Miller, R. D., L. Prakash, and S. Prakash.** 1982. Defective excision of pyrimidine dimers and interstrand DNA crosslinks in rad7 and rad23 mutants of Saccharomyces cerevisiae. Mol Gen Genet **188:**235-9.
162. **Mizuno, E., K. Kawahata, M. Kato, N. Kitamura, and M. Komada.** 2003. STAM proteins bind ubiquitinated proteins on the early endosome via the VHS domain and ubiquitin-interacting motif. Mol Biol Cell **14:**3675-89.
163. **Muratani, M., and W. P. Tansey.** 2003. How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol **4:**192-201.
164. **Nagayama, A., C. Kato, and F. Abe.** 2004. The N- and C-terminal mutations in tryptophan permease Tat2 confer cell growth in Saccharomyces cerevisiae under high-pressure and low-temperature conditions. Extremophiles **8:**143-9.
165. **Nakagawa, S., and J. M. Huibregtse.** 2000. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. Mol Cell Biol **20:**8244-53.
166. **Neumann, S., E. Petfalski, B. Brugger, H. Grosshans, F. Wieland, D. Tollervey, and E. Hurt.** 2003. Formation and nuclear export of tRNA, rRNA and mRNA is regulated by the ubiquitin ligase Rsp5p. EMBO Rep **4:**1156-62.
167. **Nijman, S. M., T. T. Huang, A. M. Dirac, T. R. Brummelkamp, R. M. Kerkhoven, A. D. D'Andrea, and R. Bernards.** 2005. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. Mol Cell **17:**331-9.
168. **Nuber, U., and M. Scheffner.** 1999. Identification of determinants in E2 ubiquitin-conjugating enzymes required for hect E3 ubiquitin-protein ligase interaction. J Biol Chem **274:**7576-82.
169. **Ogunjimi, A. A., D. J. Briant, N. Pece-Barbara, C. Le Roy, G. M. Di Guglielmo, P. Kavsak, R. K. Rasmussen, B. T. Seet, F. Sicheri, and J. L.**

Wrana. 2005. Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol Cell **19:**297-308.

170. **Okumura, A., G. Lu, I. Pitha-Rowe, and P. M. Pitha.** 2006. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. Proc Natl Acad Sci U S A **103:**1440-5.
171. **Peng, J., D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley, and S. P. Gygi.** 2003. A proteomics approach to understanding protein ubiquitination. Nat Biotechnol **21:**921-6.
172. **Perry, W. L., C. M. Hustad, D. A. Swing, T. N. O'Sullivan, N. A. Jenkins, and N. G. Copeland.** 1998. The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. Nat Genet **18:**143-6.
173. **Petroski, M. D., and R. J. Deshaies.** 2005. Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol **6:**9-20.
174. **Pickart, C. M.** 2001. Mechanisms underlying ubiquitination. Annu Rev Biochem **70:**503-33.
175. **Pickart, C. M., and D. Fushman.** 2004. Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol **8:**610-6.
176. **Polo, S., S. Sigismund, M. Faretta, M. Guidi, M. R. Capua, G. Bossi, H. Chen, P. De Camilli, and P. P. Di Fiore.** 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature **416:**451-5.
177. **Puig, O., F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, and B. Seraphin.** 2001. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods **24:**218-29.
178. **Raasi, S., R. Varadan, D. Fushman, and C. M. Pickart.** 2005. Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat Struct Mol Biol **12:**708-14.
179. **Rao, H., and A. Sastry.** 2002. Recognition of specific ubiquitin conjugates is important for the proteolytic functions of the ubiquitin-associated domain proteins Dsk2 and Rad23. J Biol Chem **277:**11691-5.
180. **Rape, M., T. Hoppe, I. Gorr, M. Kalocay, H. Richly, and S. Jentsch.** 2001. Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. Cell **107:**667-77.
181. **Rape, M., and S. Jentsch.** 2004. Productive RUPture: activation of transcription factors by proteasomal processing. Biochim Biophys Acta **1695:**209-13.
182. **Ren, J., Y. Kee, J. M. Huibregtse, and R. C. Piper.** 2006. Hse1, a Component of the Yeast Hrs-STAM Ubiquitin Sorting Complex, Associates with Ubiquitin Peptidases and a Ligase to Control Sorting Efficiency into Multivesicular Bodies. Mol Biol Cell.
183. **Rodriguez, M. S., C. Gwizdek, R. Haguenauer-Tsapis, and C. Dargemont.** 2003. The HECT ubiquitin ligase Rsp5p is required for proper nuclear export of mRNA in Saccharomyces cerevisiae. Traffic **4:**566-75.
184. **Rossi, M., R. I. Aqeilan, M. Neale, E. Candi, P. Salomoni, R. A. Knight, C. M. Croce, and G. Melino.** 2006. The E3 ubiquitin ligase Itch controls the protein stability of p63. Proc Natl Acad Sci U S A **103:**12753-8.

185. **Rossi, M., V. De Laurenzi, E. Munarriz, D. R. Green, Y. C. Liu, K. H. Vousden, G. Cesareni, and G. Melino.** 2005. The ubiquitin-protein ligase Itch regulates p73 stability. Embo J **24:**836-48.
186. **Rotin, D., O. Staub, and R. Haguenauer-Tsapis.** 2000. Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. J Membr Biol **176:**1-17.
187. **Saeki, Y., E. Isono, and E. A. Toh.** 2005. Preparation of Ubiquitinated Substrates by the PY Motif-Insertion Method for Monitoring 26S Proteasome Activity. Methods Enzymol **399:**215-27.
188. **Saeki, Y., Y. Tayama, A. Toh-e, and H. Yokosawa.** 2004. Definitive evidence for Ufd2-catalyzed elongation of the ubiquitin chain through Lys48 linkage. Biochem Biophys Res Commun **320:**840-5.
189. **Salvat, C., G. Wang, A. Dastur, N. Lyon, and J. M. Huibregtse.** 2004. The -4 phenylalanine is required for substrate ubiquitination catalyzed by HECT ubiquitin ligases. J Biol Chem **279:**18935-43.
190. **Santos, B., and M. Snyder.** 2000. Sbe2p and sbe22p, two homologous Golgi proteins involved in yeast cell wall formation. Mol Biol Cell **11:**435-52.
191. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell **75:**495-505.
192. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc Natl Acad Sci U S A **88:**5523-7.
193. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. Nature **373:**81-3.
194. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129-36.
195. **Scheffner, M., and N. J. Whitaker.** 2003. Human papillomavirus-induced carcinogenesis and the ubiquitin-proteasome system. Semin Cancer Biol **13:**59-67.
196. **Schild, L., C. M. Canessa, R. A. Shimkets, I. Gautschi, R. P. Lifton, and B. C. Rossier.** 1995. A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the Xenopus laevis oocyte expression system. Proc Natl Acad Sci U S A **92:**5699-703.
197. **Sebban, H., S. Yamaoka, and G. Courtois.** 2006. Posttranslational modifications of NEMO and its partners in NF-kappaB signaling. Trends Cell Biol **16:**569-77.
198. **Seigneurin-Berny, D., A. Verdel, S. Curtet, C. Lemercier, J. Garin, S. Rousseaux, and S. Khochbin.** 2001. Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways. Mol Cell Biol **21:**8035-44.
199. **Shcherbik, N., Y. Kee, N. Lyon, J. M. Huibregtse, and D. S. Haines.** 2004. A single PXY motif located within the carboxyl terminus of Spt23p and Mga2p

mediates a physical and functional interaction with ubiquitin ligase Rsp5p. J Biol Chem **279:**53892-8.

200. **Shcherbik, N., T. Zoladek, J. T. Nickels, and D. S. Haines.** 2003. Rsp5p is required for ER bound Mga2p120 polyubiquitination and release of the processed/tethered transactivator Mga2p90. Curr Biol **13:**1227-33.
201. **Shearwin-Whyatt, L., H. E. Dalton, N. Foot, and S. Kumar.** 2006. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. Bioessays **28:**617-28.
202. **Sherr, C. J., and J. D. Weber.** 2000. The ARF/p53 pathway. Curr Opin Genet Dev **10:**94-9.
203. **Shiba, Y., Y. Katoh, T. Shiba, K. Yoshino, H. Takatsu, H. Kobayashi, H. W. Shin, S. Wakatsuki, and K. Nakayama.** 2004. GAT (GGA and Tom1) domain responsible for ubiquitin binding and ubiquitination. J Biol Chem **279:**7105-11.
204. **Shih, S. C., G. Prag, S. A. Francis, M. A. Sutanto, J. H. Hurley, and L. Hicke.** 2003. A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. Embo J **22:**1273-81.
205. **Slagsvold, T., R. Aasland, S. Hirano, K. G. Bache, C. Raiborg, D. Trambaiolo, S. Wakatsuki, and H. Stenmark.** 2005. Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. J Biol Chem **280:**19600-6.
206. **Soetens, O., J. O. De Craene, and B. Andre.** 2001. Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1. J Biol Chem **276:**43949-57.
207. **Spence, J., R. R. Gali, G. Dittmar, F. Sherman, M. Karin, and D. Finley.** 2000. Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain. Cell **102:**67-76.
208. **Spence, J., S. Sadis, A. L. Haas, and D. Finley.** 1995. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. Mol Cell Biol **15:**1265-73.
209. **Stamenova, S. D., R. Dunn, A. S. Adler, and L. Hicke.** 2004. The Rsp5 ubiquitin ligase binds to and ubiquitinates members of the yeast CIN85-endophilin complex, Sla1-Rvs167. J Biol Chem **279:**16017-25.
210. **Staub, O., S. Dho, P. Henry, J. Correa, T. Ishikawa, J. McGlade, and D. Rotin.** 1996. WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. Embo J **15:**2371-80.
211. **Staub, O., I. Gautschi, T. Ishikawa, K. Breitschopf, A. Ciechanover, L. Schild, and D. Rotin.** 1997. Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. Embo J **16:**6325-36.
212. **Staub, O., and D. Rotin.** 1996. WW domains. Structure **4:**495-9.
213. **Staub, O., H. Yeger, P. J. Plant, H. Kim, S. A. Ernst, and D. Rotin.** 1997. Immunolocalization of the ubiquitin-protein ligase Nedd4 in tissues expressing the epithelial Na+ channel (ENaC). Am J Physiol **272:**C1871-80.
214. **Stukey, J. E., V. M. McDonough, and C. E. Martin.** 1989. Isolation and characterization of OLE1, a gene affecting fatty acid desaturation from Saccharomyces cerevisiae. J Biol Chem **264:**16537-44.

215. **Swaminathan, S., A. Y. Amerik, and M. Hochstrasser.** 1999. The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. Mol Biol Cell **10:**2583-94.
216. **Tagwerker, C., K. Flick, M. Cui, C. Guerrero, Y. Dou, B. Auer, P. Baldi, L. Huang, and P. Kaiser.** 2006. A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivocross-linking. Mol Cell Proteomics **5:**737-48.
217. **Talis, A. L., J. M. Huibregtse, and P. M. Howley.** 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J Biol Chem **273:**6439-45.
218. **Terrell, J., S. Shih, R. Dunn, and L. Hicke.** 1998. A function for monoubiquitination in the internalization of a G protein-coupled receptor. Mol Cell **1:**193-202.
219. **Tong, A. H., G. Lesage, G. D. Bader, H. Ding, H. Xu, X. Xin, J. Young, G. F. Berriz, R. L. Brost, M. Chang, Y. Chen, X. Cheng, G. Chua, H. Friesen, D. S. Goldberg, J. Haynes, C. Humphries, G. He, S. Hussein, L. Ke, N. Krogan, Z. Li, J. N. Levinson, H. Lu, P. Menard, C. Munyana, A. B. Parsons, O. Ryan, R. Tonikian, T. Roberts, A. M. Sdicu, J. Shapiro, B. Sheikh, B. Suter, S. L. Wong, L. V. Zhang, H. Zhu, C. G. Burd, S. Munro, C. Sander, J. Rine, J. Greenblatt, M. Peter, A. Bretscher, G. Bell, F. P. Roth, G. W. Brown, B. Andrews, H. Bussey, and C. Boone.** 2004. Global mapping of the yeast genetic interaction network. Science **303:**808-13.
220. **Trompouki, E., E. Hatzivassiliou, T. Tsichritzis, H. Farmer, A. Ashworth, and G. Mosialos.** 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature **424:**793-6.
221. **Varadan, R., M. Assfalg, A. Haririnia, S. Raasi, C. Pickart, and D. Fushman.** 2004. Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J Biol Chem **279:**7055-63.
222. **Verdecia, M. A., C. A. Joazeiro, N. J. Wells, J. L. Ferrer, M. E. Bowman, T. Hunter, and J. P. Noel.** 2003. Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol Cell **11:**249-59.
223. **Verma, R., L. Aravind, R. Oania, W. H. McDonald, J. R. Yates, 3rd, E. V. Koonin, and R. J. Deshaies.** 2002. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science **298:**611-5.
224. **Vu, T. H., and A. R. Hoffman.** 1997. Imprinting of the Angelman syndrome gene, UBE3A, is restricted to brain. Nat Genet **17:**12-3.
225. **Wang, C., L. Deng, M. Hong, G. R. Akkaraju, J. Inoue, and Z. J. Chen.** 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature **412:**346-51.
226. **Wang, G., J. M. McCaffery, B. Wendland, S. Dupre, R. Haguenauer-Tsapis, and J. M. Huibregtse.** 2001. Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. Mol Cell Biol **21:**3564-75.
227. **Wang, G., J. Yang, and J. M. Huibregtse.** 1999. Functional domains of the Rsp5 ubiquitin-protein ligase. Mol Cell Biol **19:**342-52.

228. **Wang, H. L., C. Y. He, A. H. Chou, T. H. Yeh, Y. L. Chen, and A. H. Li.** 2006. Polyglutamine-expanded ataxin-7 decreases nuclear translocation of NF-kappaB p65 and impairs NF-kappaB activity by inhibiting proteasome activity of cerebellar neurons. Cell Signal.
229. **Wang, M., and C. M. Pickart.** 2005. Different HECT domain ubiquitin ligases employ distinct mechanisms of polyubiquitin chain synthesis. Embo J **24:**4324-33.
230. **Wang, W., W. Zhou, L. Jiang, B. Cui, L. Ye, T. Su, J. Wang, X. Li, and G. Ning.** 2006. Mutation analysis of SCNN1B in a family with Liddle's syndrome. Endocrine **29:**385-90.
231. **Wendland, B., and S. D. Emr.** 1998. Pan1p, yeast eps15, functions as a multivalent adaptor that coordinates protein-protein interactions essential for endocytosis. J Cell Biol **141:**71-84.
232. **Wertz, I. E., K. M. O'Rourke, H. Zhou, M. Eby, L. Aravind, S. Seshagiri, P. Wu, C. Wiesmann, R. Baker, D. L. Boone, A. Ma, E. V. Koonin, and V. M. Dixit.** 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature **430:**694-9.
233. **Wilkinson, C. R., M. Seeger, R. Hartmann-Petersen, M. Stone, M. Wallace, C. Semple, and C. Gordon.** 2001. Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. Nat Cell Biol **3:**939-43.
234. **Wilkinson, K. D.** 2005. The discovery of ubiquitin-dependent proteolysis. Proc Natl Acad Sci U S A **102:**15280-2.
235. **Wilkinson, K. D., M. K. Urban, and A. L. Haas.** 1980. Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. J Biol Chem **255:**7529-32.
236. **Wong, J. J., Y. F. Pung, N. S. Sze, and K. C. Chin.** 2006. HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. Proc Natl Acad Sci U S A **103:**10735-40.
237. **Wu-Baer, F., K. Lagrazon, W. Yuan, and R. Baer.** 2003. The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin. J Biol Chem **278:**34743-6.
238. **Wu, C. J., D. B. Conze, T. Li, S. M. Srinivasula, and J. D. Ashwell.** 2006. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol **8:**398-406.
239. **Wu, X., L. Yen, L. Irwin, C. Sweeney, and K. L. Carraway, 3rd.** 2004. Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8. Mol Cell Biol **24:**7748-57.
240. **Wysocka, J., T. Swigut, H. Xiao, T. A. Milne, S. Y. Kwon, J. Landry, M. Kauer, A. J. Tackett, B. T. Chait, P. Badenhorst, C. Wu, and C. D. Allis.** 2006. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature **442:**86-90.
241. **Xu, L., Y. Wei, J. Reboul, P. Vaglio, T. H. Shin, M. Vidal, S. J. Elledge, and J. W. Harper.** 2003. BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature **425:**316-21.

242. **Yang, C., W. Zhou, M. S. Jeon, D. Demydenko, Y. Harada, H. Zhou, and Y. C. Liu.** 2006. Negative regulation of the E3 ubiquitin ligase itch via Fyn-mediated tyrosine phosphorylation. Mol Cell **21:**135-41.
243. **Yashiroda, H., D. Kaida, A. Toh-e, and Y. Kikuchi.** 1998. The PY-motif of Bul1 protein is essential for growth of Saccharomyces cerevisiae under various stress conditions. Gene **225:**39-46.
244. **Yashiroda, H., T. Oguchi, Y. Yasuda, E. A. Toh, and Y. Kikuchi.** 1996. Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in Saccharomyces cerevisiae. Mol Cell Biol **16:**3255-63.
245. **Yasuda, J., E. Hunter, M. Nakao, and H. Shida.** 2002. Functional involvement of a novel Nedd4-like ubiquitin ligase on retrovirus budding. EMBO Rep **3:**636-40.
246. **Yonashiro, R., S. Ishido, S. Kyo, T. Fukuda, E. Goto, Y. Matsuki, M. Ohmura-Hoshino, K. Sada, H. Hotta, H. Yamamura, R. Inatome, and S. Yanagi.** 2006. A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. Embo J **25:**3618-26.
247. **Young, P., Q. Deveraux, R. E. Beal, C. M. Pickart, and M. Rechsteiner.** 1998. Characterization of two polyubiquitin binding sites in the 26 S protease subunit 5a. J Biol Chem **273:**5461-7.
248. **Yuan, W., and R. M. Krug.** 2001. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. Embo J **20:**362-71.
249. **Zhang, Y., C. Chang, D. J. Gehling, A. Hemmati-Brivanlou, and R. Derynck.** 2001. Regulation of Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc Natl Acad Sci U S A **98:**974-9.
250. **Zhao, C., S. L. Beaudenon, M. L. Kelley, M. B. Waddell, W. Yuan, B. A. Schulman, J. M. Huibregtse, and R. M. Krug.** 2004. The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN-alpha/beta-induced ubiquitin-like protein. Proc Natl Acad Sci U S A **101:**7578-82.
251. **Zhao, M., M. Qiao, B. O. Oyajobi, G. R. Mundy, and D. Chen.** 2003. E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. J Biol Chem **278:**27939-44.
252. **Zheng, N.** 2003. A closer look of the HECTic ubiquitin ligases. Structure **11:**5-6.
253. **Zheng, N., P. Wang, P. D. Jeffrey, and N. P. Pavletich.** 2000. Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. Cell **102:**533-9.
254. **Zoladek, T., A. Tobiasz, G. Vaduva, M. Boguta, N. C. Martin, and A. K. Hopper.** 1997. MDP1, a Saccharomyces cerevisiae gene involved in mitochondrial/cytoplasmic protein distribution, is identical to the ubiquitin-protein ligase gene RSP5. Genetics **145:**595-603.
255. **Zoladek, T., G. Vaduva, L. A. Hunter, M. Boguta, B. D. Go, N. C. Martin, and A. K. Hopper.** 1995. Mutations altering the mitochondrial-cytoplasmic distribution of Mod5p implicate the actin cytoskeleton and mRNA 3' ends and/or protein synthesis in mitochondrial delivery. Mol Cell Biol **15:**6884-94.

256. **zur Hausen, H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer **2:**342-50.

Vita

Younghoon Kee was born in Seoul, South Korea on April 21, 1972. He entered Hankuk University of Foreign Studies (HUFS) at Yong-in, South Korea at 1992. He joined the Army in 1994 and served for 26 months before he finshed his undergraduate studies in 1999. He received his Master's degree in 2001 in Dr. Heejun Myung's lab at the same school. In the fall of 2001, he came to the U.S. and entered the CMB program at The University of Texas at Austin, and began his doctoral research in Dr. Jon Huibregtse's lab in May, 2002.

Permanent address: Yong-san, Seo-bing-go, Shin-dong-a APT #8-301
Seoul, Korea 140-240

This dissertation was typed by the author.